INTRACELLULAR LOCATIONS OF INFLUENZA VIRUS PROTEINS

by

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DECLARATION

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which this is a record has been done by myself, and all sources of information have been specifically acknowledged by means of references.

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I shall always be grateful to my parents for sustaining and encouraging me through the darkest hours and never letting me quite forget the work in progress. I should like to thank Barbara Garlick who supervised my metamorphosis.

But most of all I should like to thank this work which enabled me, through its execution at Warwick, to meet and marry Josephine Flynn.

SUMMARY

This work details the location and movement of the major influenza virus proteins throughout the process of infection.

The first section documents and analyzes the fate of proteins from the input virus on infection at 4^{o} and on subsequent incubation at 37^{o}.

The second section describes the appearance and movement of newly synthesized viral proteins both into and out of the nucleus, and details the release of virions and viral components into the tissue culture fluids.

The third section investigates the state of newly synthesized viral proteins within both nucleus and cytoplasm.

The appendix reviews the methods of cell fractionation and their criteria of purity.

ABBREVIATIONS

AMD	actinomycin D
BHK	baby hamster kidney cells
CAV	cell associated virus
CEF	chick embryo fibroblasts
cpm	counts per minute
DOC	sodium deoxycholate
DMSO	dimethyl sulphoxide
EDC(x)	Earles salt solution plus dialysed calf serum(x%)
FPV	fowl plague virus
GM(x)	Glasgow modified growth medium plus dialysed calf serum (x%)
HA	haemagglutinin
HAU	HA units
(m)hce	(million) whole cell equivalents
IC	intracellular
M	matrix protein
Metrizamide	2-(3-acetamido-5-N-methylacetamido-2, 4, 6-tri-iodobenzamido)-2-deoxy-D-glucose
MOI	mulitplicity of infection
NA	neuraminidase
NDV	Newcastle disease virus
NML	nuclear monolayer
NP	nucleoprotein
NP40	Nonidet P40
NS	non-structural
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS C/M	PBS with 0.5 mM Ca Cl_2 and 0.5 mM $MgCl_2$
pfu	plaque forming unit
pol	RNA polymerase

/continued

RDE	receptor destroying enzyme
cRNA	virus RNA complementary to the viral genome i.e.(+) sense
vRNA	viralgenome (-) sense
RNP	ribonucleoprotein
SDS	sodium dodecyl sulphate
SFV	Semliki forest virus
TCA	trichloracetic acid
TCF	tissue culture fluids
tris	Tris(hydroxymethyl) aminomethane
VSV	vesicular stomatitis virus

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GENERAL INTRODUCTION

1. Preamble

A pandemic of influenza was first documented in 1510 (Francis and Maassab, 1965) though the virus was not isolated until 1933 by W. Smith and co-workers (Smith et al., 1933). The disease, caused by type A influenza, is found in all parts of the world and in a pandemic year between 30 and 80% of the entire human population may contract the disease in some form. Since 1933 it has been studied extensively and yet despite enormous advances in the understanding of virus structure, virus replication and immunology, there has been no substantial curtailment of the disease itself.

The occurrence of the disease appears to follow a pattern in which a world-wide epidemic, a 'pandemic', is followed by several years in which the disease occurs at much lower levels. Both of the surface antigens of influenza virus, the haemagglutinin (HA) and the neuraminidase (NA) undergo radical changes, named antigenic 'shift', and relatively minor but frequent changes, called antigenic 'drift'. Pandemics occur after a shift and are followed by several years of antigenic drift. For example, the Asian strain of 'flu'(H2.N2) persisted until 1968, when a new strain was isolated in Hong Kong containing an entirely different haemagglutinin but a similar neuraminidase (H3.N2). A pandemic followed in the winter of 1969. Subsequently the H3 has undergone the antigenic drift.

How do these antigenic changes arise ? It seems that drift occurs due to the selection pressure exerted by neutralizing antibody upon mutations arising in the antigenic sites. Such drift has been mimicked in laboratory conditions (Laver and Webster, 1976). Shift is now thought, from circumstantial evidence, to arise by a quite different mechanism involving two features in combination unique to influenza A viruses: the recombination or, more accurately, 'reassortment' process and the presence of an animal reservoir.

The influenza genome is segmented and the infection of a host cell with more than one strain of virus results in reassortment of

the segments to produce new strains (e.g. Hswl.Nl x Havl.Neql = Hswl.Neql + Havl.Nl (Webster et al., 1971)).

The animal reservoir, as its name implies, provides a stock of influenza antigens with which human influenza type A can exchange to produce the new antigenic strain, the 'shift' process. Pigs and horses are implicated but more probably the bird population is most important. Kilbourne (p. 514, 1975) has advanced the view that when such an exchange has taken place, the virus only produces pandemic disease in man if an ecologic niche is available, i.e. sufficiently high antibody levels in the population to the current strain must be present, thus providing a selective advantage to the new 'pandemic candidate'. There may be a number of such viruses awaiting the opportunity to be introduced into the human population.

As the span of precise biochemical and serological documentation of influenza virus pandemics increases, it becomes possible to observe any re-emergence of HA or NA antigen in a new pandemic when herd immunity no longer precludes it from the human population. The isolation of A/New Jersey/76 recently (WHO, 1976), with antigens of 'classical' swine influenza virus (Hswl.Nl), which is thought to have caused the 1918 pandemic, may be one such example, and the presence in the sera of elderly people of antibodies to an HA similar to Hong Kong but not to the NA suggest that the pandemic at the turn of the century may have had a Hong Kong type HA (H3) but a different NA.

The ubiquity of the disease coupled with its enormous cost in economic terms (in the USA $1.7-3.9 billion/year in 1963, 66, 69 - Kavet, 1973) justify the research effort into influenza, but there are other reasons why the field is so attractive. Influenza virus replication provides a useful model to study the processes of the mammalian cell. Whereas the latter has a genome with coding capacity for around 10^7 proteins, there are just 8 genome segments in the virus and this, together with its requirement for a host nucleus in replication (see Introduction, part 6), suggestsa unique dependence on host systems. This makes it an excellent model to probe the processes of the host cell.

It was in order to try to catalogue and illuminate some of the movements of viral components into and out of the nucleus and within this organelle that this study was initiated. It was hoped that correlation of these movements with events in the virus multiplication cycle would result in a better understanding of the roles of the viral proteins and the mechanisms and structures involved in virus infection. Section 1 deals with the fate of the input viral components, whilst Section 2 details the movements of newly synthesized proteins within the cell and follows these proteins into the tissue culture fluids. In Section 3 the structures in which newly synthesized proteins are found in nucleus and cytoplasmare investigated. Finally, in the appendix, there is a detailed characterization and analysis of methods of nuclear fractionation.

2. The Structure of Influenza Virus and the Functional Role of its Components.

Influenza viruses consist of three distinct antigenic types, A, B and C, which are determined by the internal ribonucleoprotein antigen. Man is the only host of B and C types, whilst A type is also found in other animals Influenza A viruses are by far the most closely studied of the types and are responsible for the pandemics described in part 1.

The influenza viruses are spherical or filamentous particles with a diameter of 80-120 nm. They possess an envelope of lipids from which the spike-like surface projections or peplomers protrude to the exterior. Inside the particle is a single-stranded RNA genome of MW 6 x 10^6 daltons in eight segments, which is complementary to the viral messenger RNA found in infected cells. The virion contains eight polypeptide species of molecular weight from 25-91 k daltons each. In addition, one or possibly two virus induced non-structural proteins are found only in infected cells.

2A Morphology

Electron microscopy reveals the laboratory adapted virus to be an irregular spherical-shaped particle of 80-120 nm, though in recently isolated strains abundant filamentous particles of up to 1,000 nm have been found (Schulze,1973).Negatively stained particles display a coat of densely

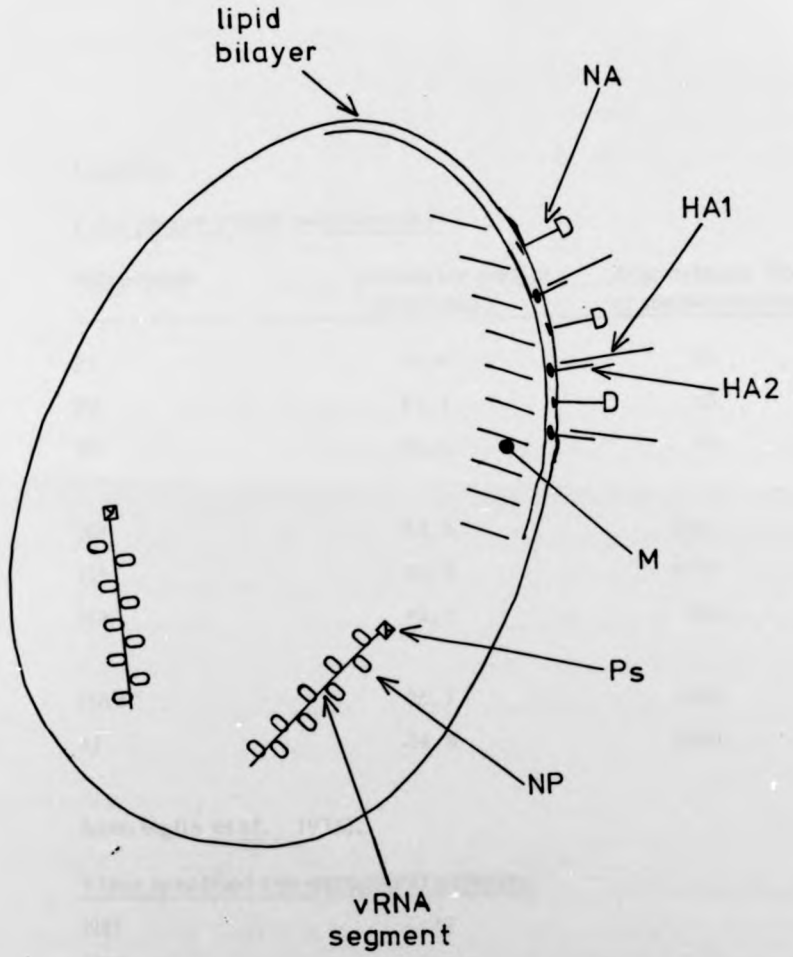

Fig. I/1 Diagrammatic representation of influenza virus showing major components

Table I/1

Fowl plague virion polypeptides

Polypeptide	Molecular weight (k daltons)	Approximate No. of molecules/virion
P1	95.9	15
P2	87.1	15
P3	85.1	15
NP	52.3	1000
HA1	44.8	1000
NA	42.1	200
HA2	26.7	1200
M	24.8	2400

from Inglis et al., 1976).

Virus specified non-structural proteins

NS1	~ 23
NS2	~ 11

arranged radial projections which comprise the haemagglutinin and neuraminidase components of the virus. Nermut and Frank (1971) have suggested that influenza virions have a more ordered structure which they called a 'plastic icosahedron'. Their freeze-etching procedures showed that there was very little pleomorphism and that the spikes are arranged in pentagonal and hexagonal arrays.

The ribonucleoprotein appears to consist of long filaments (up to 2 μm) which consist of a 9 nm diameter fibre in a double helix of 50-60 nm diameter (Almeida and Brand, 1975).

A diagrammatic representation of influenza virus is presented in Fig. I/1. The NA and HA glycoproteins which contain host-specified carbonydrate are present on the outer membrane surface as distinct spikes embedded in the lipid bilayer of the virus which is also host-specified. Within the envelope, matrix (M) protein is present either as a shell just beneath the membrane (Lenard et al., 1974) or as a plastic 'filler' between membrane and RNP core (Laver, 1973) or more closely associated with the RNP (Reginster and Nermut, 1976). The ribonucleoprotein comprises the RNA of the virus, closely associated with NP and probably all three P proteins.

2B Polypeptides of the Virus

The virus contains eight polypeptides (Table I/1) from Compans et al., 1970a; Skehel and Schild, 1971: Inglis et al., 1976, three of which (HA1, HA2, NA) are glycosylated (Schulze, 1970; Compans et al., 1970a). An additional glycoprotein (HA) is also observed in the virions of some strains of virus grown in some cell systems (e.g. WSN in MK cells) but is present in variable amounts. This protein has been shown to be cleaved by host cell systems or by serum proteases upon contact with cell activators (Lazarowitz et al., 1973) to yield HA1 and HA2.

2C Function of Viral Components: Role of the Proteins

The Nucleocapsid

The nucleoprotein of the virus is found closely associated with the viral genome in an RNA-protein complex of about 10% RNA and 90% protein (Pons et al., 1969). The P proteins are also found in association with

this complex both in virions (Bishop et al., 1972) and in the infected cell (Caliguiri and Compans, 1974) although they are more easily removed during purification than NP (Schulze, 1973). Several distinct sizes of ribonucleoprotein can be separated (three by Duesberg, 1969; five by Pons, 1971) on velocity gradient centrifugation which are of the same diameter but are of considerably different lengths (Compans et al., 1972). These were thought to correspond to different segments of the genome, individually packaged. This situation needs re-examining since the genome is now known to comprise 8 RNA segments. The NP, of which there are about 1,000 copies per virion, forms the protein subunit of the nucleocapsid (Duesberg, 1969; Pons et al., 1969). It is antigenically stable and forms the basis of the classification of influenza into A, B and C viruses. It displays considerable affinity for c and vRNA (Scholtissek and Becht, 1971), and can be visualized in a complex structure with the RNA under the electron microscope (reviewed by Schulze, 1973).

The nucleocapsid structure is active in RNA synthesis in both virions (Bishop et al., 1972; Rochovansky, 1976; Content et al., 1977) and in the infected cell (Compans and Caliguiri, 1973; Caliguiri and Compans, 1974) and can produce a transcipt from which all the proteins can be translated (Content et al., 1977). Both the P proteins (P2 was not resolved from P3 in these studies) and NP were present in the virion complex, and though early reports suggested that the P proteins were not involved in polymerase activity in complexes from the infected cell (Compans and Caliguiri, 1973), a later report confirmed their presence (Caliguiri and Compans, 1974). Palese et al. (1977), using temperature sensitive (ts-) mutants of influenza, found two groups (I, III) which failed to synthesize cRNA at the non-permissive temperature. These had altered RNA's coding for P1 and P3 and were rescued only by recombinations with the wild type involving these RNA's. The function of P1 and P3 seems fairly certain to be in the synthesis of RNA, though their precise role in initiation or elongation has yet to be elucidated. It is thought that P2 may function similarly to P3 but as they have only recently been resolved (Inglis et al., 1976; Lamb and Choppin,1976), this is still uncertain. The ratio of the P proteins is about

1:1:1 and there are approximately 15 molecules of each in the virion (Inglis et al., 1976).

It is now known how the individual RNP's are sorted and arranged in the virion particle, but there has been a suggestion that the particle may enclose a random 10 or 11 RNP's (Hirst, 1962; Hirst and Pons, 1973) which may explain the low PFU to particle ratio found in some strains. However, this ratio varies considerably between different virus strains and is thus not an all-embracing solution. This is considered further in Part 5 of the Introduction.

The Role of the Proteins: the Viral Envelope and its Components

The viral envelope consists of a lipid bilayer (Landsberger et al., 1970, 1973) whose appearance and composition is very similar to that of the host membrane from which it was derived (Compans and Dimmock, 1969). It contains no host protein (Holland and Kiehn, 1970) but does contain host-specified carbohydrate and lipid (Kates et al., 1961, 1962) though there are restrictions on the carbohydrate content which preclude the incorporation of neuraminic acid (Klenk et al., 1970).

The Role of the Proteins: Haemagglutinin

The major glycoprotein in the virus membrane is the haemagglutinin which comprises 15% of the virus protein (Inglis et al., 1976). It appears to be present as a radial spike with triangular cross-section consisting of a trimer of HA molecules either intact or, as a result of proteolytic cleavage, present as two polypeptides (HA1 and HA2) which are linked by disulphide bonds (Laver, 1973). In the latter arrangement the HA2 molecule is thought to be involved in locating the haemagglutinin in the virus membrane for two reasons. Protease treatment removes only HA1 intact (Compans et al., 1970a) and secondly, solubilized spikes lack only a small part of the HA2 molecule (Brand and Skehel, 1972). This locating function may be accomplished by a hydrophobic region in the HA2 polypeptide chain as suggested by the aggregation by one end of detergent-isolated spikes after removal of the SDS (Laver and Valentine, 1969) and the lack of aggregation in protease-isolated spikes lacking a small portion of HA2 (Brand and Skehel, 1972).

This virus glycoprotein is responsible for haemagglutination caused by its affinity for erythrocyte receptors and is responsible for the attachment of the virus particle to receptor sites (see Introduction, part 3A).

However, the observation that cleavage of HA into HA1 and HA2 in some influenza A strains caused no detectable alteration of virus binding to fetuin, adsorption to MDBK cells or haemagglutination whilst increasing infectivity by as much as 100-fold (Lazarowitz and Choppin, 1975; Klenk et al., 1975) suggest that the HA molecule may also have some other function.

Role of the Proteins: Neuraminidase

This glycoprotein is observed under the electron microscope to be shaped like a spike with a knob on one end. Rosettes are formed, with knobs outermost from detergent isolated (but detergent stable) molecules when the detergent is removed (Laver and Valentine, 1969). The neuraminidase complex is a tetramer of molecular weight 200-250 k daltons (Kendal and Eckert, 1972; Lazdins et al., 1972).

Its enzymic activity catalyzes the removal of terminal N-acetyl neuraminic acid from specific glycoprotein substrates. Although it has been suggested that NA mediates the release of virus from the host cell surface after multiplication (Gottschalk, 1966), monovalent antibodies which inhibit the enzymic activity are found not to interfere with virus release (Becht et al., 1971). The NA does appear to remove neuraminic acid from host carbohydrate incorporated in the virus particle and has thus been implicated in virus maturation (Klenk et al., 1970). Conclusive evidence that it prevents extensive aggregation of virions has been obtained by the following. Klenk et al. (1970) had shown that wild type virions have no neuraminic acid in their carbohydrate but in ts mutants defective in NA, Palese et al. (1974) found that neuraminic acid was present and that aggregation of particles occurred. This effect was overcome by the action of exogeneous bacterial neuraminidase. Furthermore, when FANA (2, deoxy - 2, 3-dehydro-N-trifluoroacetylneuraminic acid), an inhibitor of NA, was present during the virus multiplication cycle, progeny virus aggregated in the medium (Palese and Compans, 1976). This virus was found to contain sialic acid residues in its envelope which acted as receptors for the HA of adjacent particles.

Role of the Proteins: Matrix

This protein, the most abundant of the virion, is found between the virus envelope and the nucleocapsid and appears as a dense area under electron microscopy. Compans et al. (1972) have suggested that the 4-6 nm thick layer of matrix protein is in close interaction with the lipid bilayer. Laver (1973) and Schulze (1973) envisaged the protein as a filler which supports the virus membrane and determines the shape. Cores produced by DOC treatment, which were free of lipid and the viral glycoproteins, were found to have a shell of M protein surrounding the nucleocapsid (Reginster and Nermut, 1976), suggesting that matrix protein may be more closely associated with the ribonucleoprotein. Apart from its (presumably) structural role in the virion, a role for the matrix protein in virus assembly has also been suggested (Choppin et al., 1972; Compans and Choppin, 1975). This consists firstly of the recognition site, which provides the signal enabling nucleocapsid to align with specific areas of the cell membrane budding virus,and secondly they suggested that it might deny access by host cell proteins to this area of cell membrane.

Role of the Proteins: Non-structurals

The virus also specifies one or two proteins which are not incorporated into the virion (Dimmock, 1969; Skehel, 1972). These have molecular weights of 23 k daltons (NS1) and 11 k daltons (NS2). Little is known of their function, but NS1 accumulates in the nucleolus (Dimmock, 1969; Krug and Soeiro, 1975) and although it is found in association with polysomes in the cytoplasm (Pons, 1972; Compans, 1973) this association is thought to be artefactual, or at least not in a ribosomal protein type interaction (Krug and Etkind, 1973). NS2 is not regularly found (Krug and Etkind, 1973; Inglis et al., 1976); it is present in different amounts in different cell lines (P.D.Minor, personal communication) and runs on PAGE at a position close to the buffer front. Thus it is not clear whether NS2 is a genuine viral protein.

2D. The Viral Genome: Sense

Following Baltimore's (1971) classification of animal viruses centred on the relationship between the viral genome and messenger RNA, evidence rapidly accumulated to indicate that the myxoviruses belonged to group V through having an RNA genome complementary to mRNA (negative stranded viruses). The discovery of a virion RNA-dependent-RNA polymerase, one of the central predictions of the

classification, had just been reported (Chow and Simpson, 1971), and Pons (1972) was soon to show that polysomal virus specified RNA was almost exclusively complementary to the RNA found in the virion. This work was extended by Glass et al. (1975) who showed that the RNA in association with polysomes was complementary to vRNA, labelled rapidly with [^{3}H] -uridine and contained poly A.

Conclusive proof of the negative sense of vRNA came with the translation of cRNA into the viral proteins. This was first reported for M by Kingsbury and Webster (1973), and later workers progressively extended this to the synthesis of the other proteins (Etkind and Krug, 1975; Content, 1976;Ritchey and Palese, 1976; Inglis et al., 1977; Stephenson et al., 1977). The virion RNA was found to direct the synthesis of a polypeptide of molecular weight similar to NP (Seigert et al., 1973; Content, 1976; Tekamp and Penhoet, 1976), but when this was characterized by tryptic peptide analysis, it was found to be quite different from NP (Tekamp and Penhoet, 1976). Discrete products of low molecular weight have been observed, but these do not correspond to any of the virus proteins (Etkind and Krug, 1975; Stephenson et al., 1977). Recently a coupled transcription/translation system has been described, dependent absolutely on RNA transcription (Content et al., 1977; P.D. Minor, unpublished results) in which cRNA is transcribed from vRNA of disrupted virions by the virion transcriptase and this cRNA acts as a template for the synthesis of the viral proteins in a reticulocyte lysate system. Recently Content reported that P1, P2 and 3, NP, M, NS1 and HA had been identified using a modified lysate together with NWS which has a more efficient polymerase than FP/R (Content at Negative Strand Virus Symposium, Cambridge, 1977).

The Viral Genome: Segmented Nature

Following the discovery of very high recombination rates between different strains of influenza viruses (Simpson and Hirst, 1961; Hirst, 1962), it was proposed that the virus genome was in segments which, in mixed infections, could be reassorted (rather than recombined in the 'classical' sense). This was supported by the multiplicity reactivation

data of Barry (1961) which suggested that six separate functional units are involved and was followed by biochemical data which showed the size of the RNA to be 7-9S and 18S rather than the predicted 38S (Davies and Barry, 1966). PAGE of labelled RNA showed first 5 segments (Pons and Hirst, 1968) but later 8 segments of single stranded RNA were visualized as the resolution of such techniques improved (McGeoch et al., 1976; Pons, 1976). The possibility that the genome was broken up during extraction, possibly at points of weakness in the molecule, was ruled out by the demonstration that the 5' end of all segments began pppAp, that they contained base sequence differences (Young and Content, 1971) and that the 3' terminal was U-OH (Lewandowski et al., 1971).

It is not known how these segments are packaged into the virion. Pons (1970) suggested that the NP protein might hold the segments together in a continuous 'string', thus ensuring a complete selection of segments, but this implies a complex specificity for NP in selecting the segments. Most investigators found the viral RNP to be present in short segments (Pons et al., 1969; Schulze et al., 1970; Compans et al., 1972), but there have been reports of a longer strand observed in disrupted particles (Li and Seto, 1970; Almeida and Brand, 1975). The ratio of the segments is approximately unity (McGeoch et al., 1976).

The Viral Genome: Assignment of RNA segments

The assignment of RNA segments to code for virus proteins was initially made on the basis of size (Inglis et al., 1976), the coding capacity of the three largest segments being adequate to code for the P proteins, the intermediate for HA, NP, NA and the smallest segments for M and NS1. Definitive assignment came from the analysis of recombinants of PR8 and HK which had RNA's and proteins of different mobilities, allowing the assignment of NP, M and NS1 to 5, 7 and 8 respectively (Ritchey et al., 1976); HA and NA to 4 and 6 (Palese and Schulman, 1976); P1 and P3 to 2 and 1 respectively (Palese et al., 1977). An independent assignment by an elegant technique devised by Inglis et al. (1977) in which RNA-RNA hybridization of individual virion RNA segments with total cRNA suppresses translation of specific mRNA's has shown a similar assignment.

3. Influenza Virus Multiplication Cycle

A. Early Events in the Infection of Cells

The process of entry of the virus into the host cell has been divided into a series of stages: collision, attachment, penetration (defined as the development of resistance to external treatments such as acid and antiserum) and uncoating. The latter is a two-stage process for enveloped viruses, the first being removal of the viral envelope, the second stage the rendering functional of the viral nucleic acid.

Early Events: Collision

The collision of the virus with the cell takes place by Brownian motion. The chance of a successful collision (defined as leading to attachment) is very low, and it has been calculated that only one attachment event occurs in 10^4 collisions (Lonberg-Holm and Philipson, 1974).

Early Events: Attachment

Attachment depends on some configuration of the virion surface (virus receptor) and its complement on the cell surface, it is perhaps worth straying into the teleological to point out that the cell surface receptor presumably has some function for the benefit of the cell, and that it serves incidentally as the means of access for the virus.

(a) The virus receptor

Of the two envelope glycoproteins of influenza, the haemagglutinin molecule is implicated as receptor by monospecific antibody inhibition (Kilbourne et al., 1968). The inhibition of neuraminidase has no effect on virus entry and selective proteolysis of the neuraminidase does not result in diminution of infectivity (Schulze, 1970). However, even this point is not unequivocal for Dourmashkin and Tyrrell (1974) observed, in a study by electron microscopy, that antineuraminidase antiserum does not prevent attachment but does inhibit penetration and would thus be expected to reduce infectivity. Infectivity is reduced by treatment of the virus with a whole range of sialoglycoproteins which bind haemagglutinin (Shen and Ginsburg, 1968) including a sialomucoprotein.

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This implies, perhaps, a role for the viral neuraminidase in freeing influenza from such entities which occur in the respiratory tract. Klenk et al. (1972) showed that the glycosyl moiety on the haemagglutinin was probably necessary to receptor function since nonglycosylated HA was unable to bind erythrocytes. However, the demonstration that cleavage of HA in the viral membrane of WSN leads to 100-fold increase in infectivity though the haemagglutinin activity is unchanged (Lazarowitz and Choppin, 1975; Klenk et al., 1975) suggests a second role for the HA in some step subsequent to attachment.

(b) The cell receptor

The cell receptor for influenza virus has been shown to have terminal neuraminic acid, the integrity of which is essential for virus attachment (Suttajit and Winzler, 1971), but it has still not been proved unequivocally to be a sialoglycoprotein. In early work (see Hoyle, 1968) RDE (receptor-destroying enzyme) later identified as a sialidase, was used to treat cells prior to their exposure to virus and rendered the cells resistant to infection thus implicating sialic acid as a receptor. Sialoglycoproteins (α or Francis inhibitors) when added to virus reversibly inhibit its subsequent attachment to cells, but a different class of proteins, the β or Chu inhibitors, also inhibit attachment, suggesting that other receptors may also be involved. The non-specific attachment of myxoviruses to glass and other inert materials (Allison and Valentine, 1960) also indicate that several 'levels' of attachment may be possible.

(c) The attachment process

Myxovirus attachment results finally in a firm bond of viral HA to cell sialic acid, but at some stage, possibly early on, electrostatic forces are involved (Huang, 1974). Polyanions inhibit the attachment of FPV (Allison and Valentine, 1960), an effect which can be overcome by the addition of polycation DEAE-dextran (Takemoto and Fabisch, 1963). However, the pH dependence of attachment of myxoviruses shows a broad optimum, encompassing neutrality, which suggests that electrostatic forces are not vital to optimum binding.

Stephenson, Hudson and Dimmock (in press) found that the attachment rate was largely unaffected by temperature, although the plateau level of attachment at 4° was only half that at 20°. In an early study, Ishida and Ackermann (1956) reported that influenza exhibited two stages of attachment. Both were temperature independent, but the first was sensitive to RDE, acid and antibody and was reversible whilst the second stage was sensitive only to antibody and was irreversible. Perhaps virus which elutes off at 37° by the action of NA (Webster and Darlington, 1969) is trapped at the first stage of this process.

(d) Theories of attachment

It appears likely that the virus is initially loosely bound to the cell and that this interaction proceeds through several stages to a firm attachment. The transfer between these stages may be temperature dependent. In the first stages an electrostatic interaction may hold virus and cell together, subsequently a lock and key type complex may occur between the viral HA and cell receptor, producing the irreversible attachment with the resistance to sialidase outlined by Ishida and Ackermann (1956). This stage may also trigger the penetration mechanism.

An alternative model put forward by Lonberg-Holm and Philipson (1974) relates the tightness of the bond to the number of virus-cell receptor unions. Initially few receptors are linked but lateral movement of further cell receptors in the two dimensional fluid membrane of the cell towards the virus increases this original number and thus strengthens the attachment. This movement is inhibited by low temperature. If the number of cell receptors is high, then the attachment process is independent of temperature for there will be sufficient receptors immediately adjacent to the virus to form a firm bond, whilst if the receptors are sparse the attachment will be inhibited at low temperatures when the membrane fluidity is reduced. Thus the variation in temperature dependence of different virus-cell systems may be accounted for by the number of cell receptors present. The proteolytic enhancement of virus attachment (Marchosi et al., 1971) is explained, and the variation

in attachment efficiency of viruses with different batches of CEF (Stephenson, Hudson and Dimmock, in press) may be due to differing sialic acid content of the cell batches.

3A Early Events: Penetration

(a) Problems in investigation

The principle investigative tool of this stage of virus entry is electron microscopy. Unfortunately this powerful and elegant technique does not lend itself readily to quantitation. It cannot determine whether the observed virus particle is leading to a productive infection whichis clearly necessary for animal viruses, where in some cases only one particle in 1,000 is infectious. Artefacts can arise due to tangential sectioning as pointed out by Dourmashkin and Tyrrell (1974) who found that electron micrographs of thin tangential sections of virus and cell could appear to show fusion of membranes when the stage was tilted. Morgan et al. (1968) were moved to conclude that it was impossible to decide between rival theories of virus penetration by electron microscopy alone. Nevertheless, this technique has permitted the derivation of different schemes of penetration.

(b) Penetration by endocytosis

Fazekas de St. Groth (1948) suggested that the entry of virus occurred by an endocytotic event in which the whole virus was engulfed in a pinocytotic vesicle, a process which he named viropexis. Recently micropinocytosis, a process which occurs independently of temperature (Allison and Davies, 1974), was suggested (Stephenson, Hudson and Dimmock, in press) to account for the high rates of penetration of influenza observed at 4°.

(c) Penetration by fusion of membranes

In the major alternative to viropexis, it is argued that the envelope of the virus fuses with the cell membrane, releasing the viral core into the cytoplasm. Thus both penetration and the first stage of uncoating are achieved in a single process. Hoyle et al. (1962) first described such a process in artificially produced cytoplasmic vesicles and this work was comprehensively amplified using competent host cells by Morgan and Rose (1968).

(d) Penetration by Paramyxoviruses

Although Apostolov and Almeida (1972) presented compelling evidence that Newcastle disease virus can fuse with an erythrocyte, this cannot necessarily be extrapolated to infection. In contrast to this, agents which enhance pinocytosis were found to increase the uptake of NDV (Durand et al., 1970). This is a method which avoids the pitfalls of electron microscopy but is correspondingly less direct.

Sendai virus has been shown to fuse with cell membranes by electron microscopy (Morgan and Howe, 1968), with cell autoradiography (Zhdanov et al., 1963) and by correlating cell fusing ability of the virus with its infectivity in different cell systems (Scheid and Choppin, 1974). In the latter, the authors showed that the smaller glycoprotein of Sendai virus (F) was involved in the haemolysin and cell fusing activities. F, they found, was obtained from an inactive precursor F_o by a proteolytic cleavage accomplished by the host cell. The ability of the host cell to effect this cleavage correlated well with the infectivity of the virus produced. This represents ingenious evidence that paramyxoviruses penetrate by fusion.

(e) Penetration by myxoviruses

Morgan and Rose (1968) found that 85% of input influenza virus penetrated by fusion in the first 15 min at 37^{o}, while Dourmashkin and Tyrrell (1970, 1974) found evidence only for pinocytosis. Evidence that influenza could penetrate CEF cells at 4^{o} (Stephenson, Hudson and Dimmock, in press) showed that 50-80% of virus became resistant to neutralization by antibody and to treatment with acid after 15 min infection. This suggests that micropinocytosis is the method of entry, at least at this temperature. Furthermore, Stephenson, Hudson and Dimmock (in press) find that there is considerable variation in the penetrating ability of influenza at low temperature in different batches of CEF.

It seems possible that fusion and micropinocytosis may both be involved in virus penetration, the significant process depending on the temperature of infection and the host cell membrane. In support of this conjecture is the almost universal observation of both fusion and endocytosis of virus particles in electron microscope studies (though with different kinetics), and the

demonstration with paramyxoviruses of significant penetration by micropinocytosis at 4° (Stephenson, Hudson and Dimmock, in press) and by fusion at 37° (Scheid and Choppin, 1974).

3A Early Events

Uncoating

(a) Stages of uncoating and their investigation

Uncoating of the enveloped viruses can be regarded in theory as a two stage process: first, the removal of the viral membrane; second, making the nucleic acid function in the first stages of multiplication (Long and Olusanya, 1972).

The viropexis theory (Fazekas de St. Groth, 1948) at first envisaged a fusion of the endocytotic vacuole containing virus with cell lysozomes and partial digestion of the viral and vacuolar membrane to liberate the viral core. However, it seems more likely that pinocytotic vesicles might fuse with internal membranes of the cell and release virus. Dourmashkin and Tyrrell (1974), in an electron microscope study, observed virus release from vacuoles without the aid of lysozomes. Presumably some degradative action is required since it has been found that inert particles, although taken up by endocytotic processes similar to those for viruses, remain in the pinocytotic vesicles (Epstein et al., 1966). Dourmashkin and Tyrrell (1974) also observed fusion of viral with vacuolar membranes which would expel the viral core into the cytoplasm.

If the virus fuses with its host cell membrane, then this incorporates the first stage of uncoating and liberates the free viral core into the cytoplasm. Thus the theories of penetration have only the second stage of uncoating in common.

Uncoating of the viral core is difficult to investigate since it is not known what sub-viral entities might occur and one is guessing at suitable isolation procedures. This stage has been studied (Nermut, 1970; Skehel, 1971; Rochovansky, 1976, for influenza viruses) by the analogy between uncoating and the species produced by detergent and proteolytic action in vitro. Both structural and functional investigations are possible.

(b) Uncoating of myxoviruses

Since myxoviruses contain an RNA dependent RNA polymerase (Chow and Simpson, 1971) capable of transcribing their genome (Bishop et al., 1971; Content

et al., 1977), it seems likely that the proteins retained in the viral core are those necessary for transcription. Recently Rochovansky (1976) has isolated a viral RNP containing two P proteins, the nucleoprotein and the RNA, which appears to produce cRNA. Studies by cell fractionation using both conventional and nuclear monolayer techniques (Stephenson and Dimmock, 1975; Hudson, Flawith and Dimmock, in press), and using cell autoradiography (Armstrong and Barry, 1975) have shown that the input viral RNA moves rapidly to the nucleus. The site of initial transcription has been shown to be in the nucleus (Armstrong and Barry, 1974) though it was also observed in the cytoplasm by Stephenson and Dimmock (1975). Recent studies on the fate of labelled input virus have suggested that a viral RNP is transported to the nucleus even at 4^{o} (Hudson, Flawith and Dimmock, in press).

For most of the remainder of this brief review of the multiplication cycle of influenza virus, it will clarify the presentation to separate the description of the processes involving RNA from those involving the proteins until the stage of virus assembly and budding.

3B Influenza Virus Multiplication Cycle: RNA Synthesis

An illustration of some of the processes of the virus RNA is presented in Fig. I/2. After uncoating, the vRNA must undergo transcription, both to form a template for the subsequent replication to form progeny vRNA and to produce mRNA for translation. Also illustrated is the phenomenon of amplification in which either newly synthesized polymerase produces more transcripts from the original vRNA or replication produces more vRNA from which cRNA can be transcribed. Asymmetric protein synthesis results from selective amplification of the synthesis of some mRNA's (Glass et al., 1975).

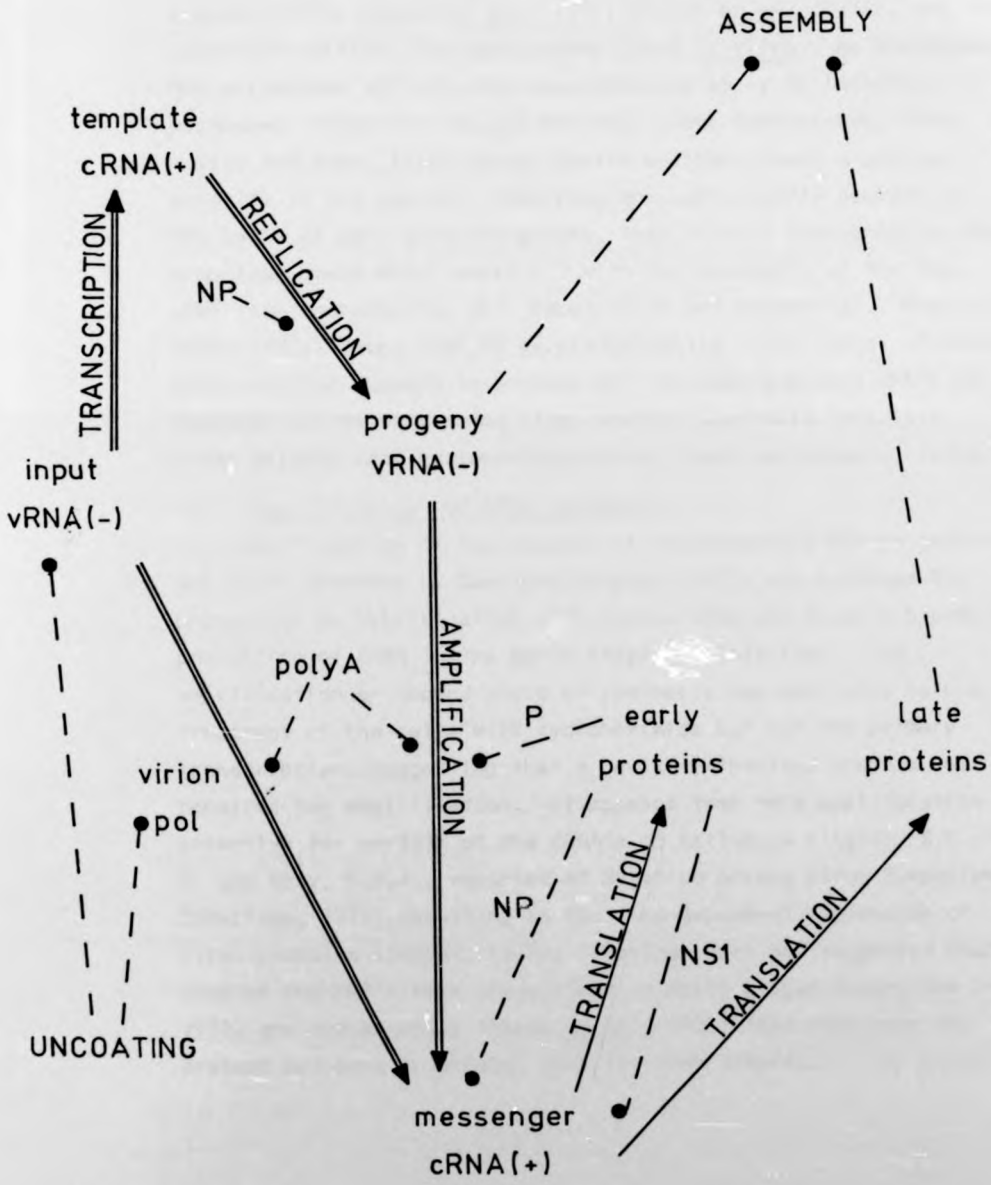

Fig. I/2 The processes of virus RNA in multiplication

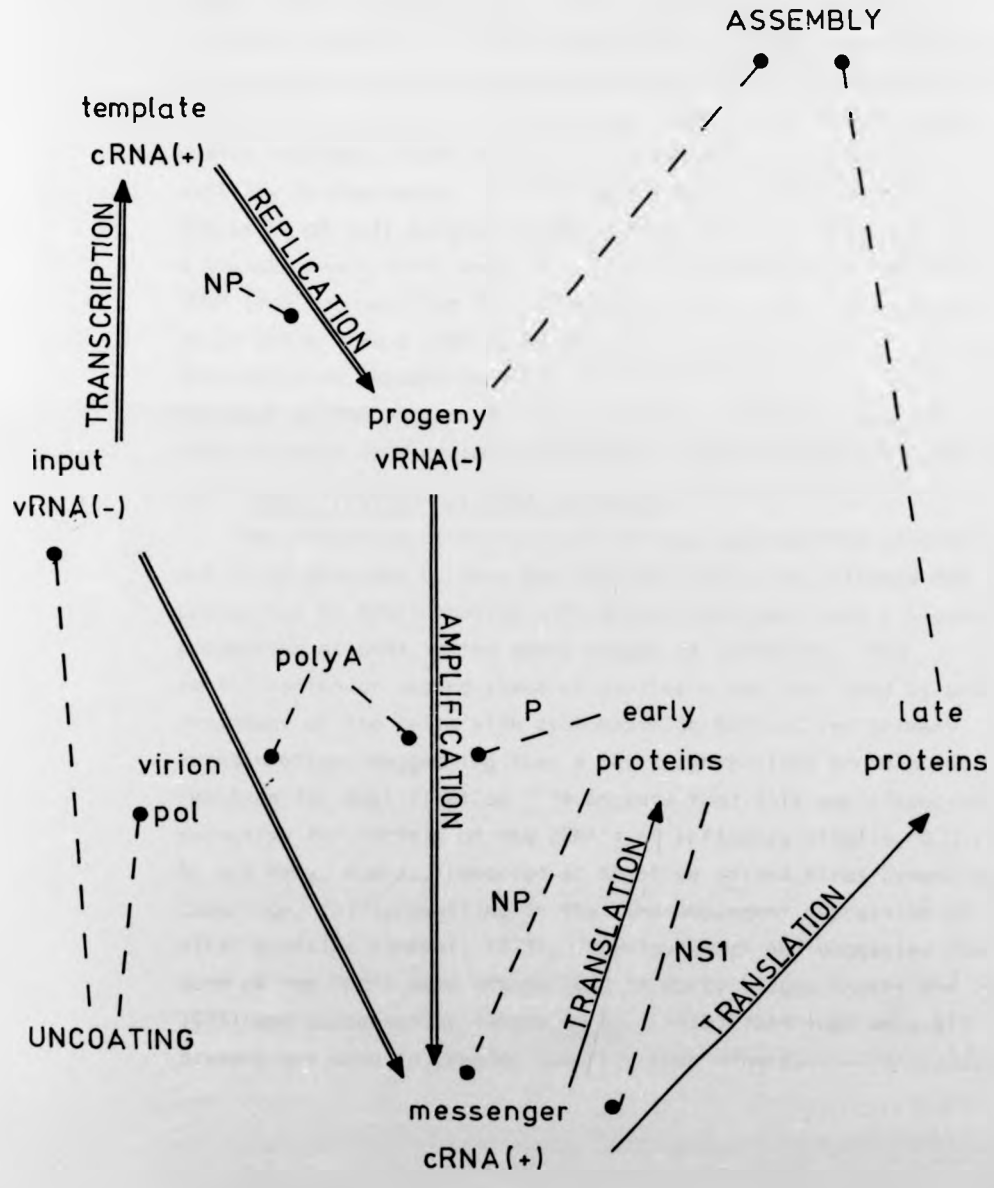

Fig. I/2 The processes of virus RNA in multiplication

(a) Synthesis of complementary RNA(+)

Primary transcription involves the virion polymerase (Chow and Simpson, 1971; Penhoet et al., 1971; Bishop et al., 1971), and, as described earlier, has been accomplished in vitro. An RNA-dependent-RNA polymerase activity has been observed early in infection in microsomal fractions (Ho and Walters, 1966; Scholtissek, 1969; Hastie and Mahy, 1973) though Hastie and Mahy found a similar activity in the nuclei. Armstrong and Barry (1974) decided, on the basis of cell autoradiography, that initial transcription was a nuclear event which would fit with the movements of the input vRNP (see Introduction 3A: Penetration and Uncoating). However, Avery (1974) found cRNA to be predominantly cytoplasmic. Primary transcription appears to produce all the complementary RNA's as revealed by the pattern of viral protein synthesis immediately after release from cycloheximide block (Lamb and Choppin, 1976).

(b) Amplification of cRNA synthesis

Amplification of the process of complementary RNA production was first observed by Bean and Simpson (1973) who followed RNA production by hybridization with excess vRNA and found a biphasic production of cRNA in the early stages of infection. The amplification or second phase of synthesis was abolished by pre-treatment of the cells with cycloheximide but not the primary transcription, suggesting that a newly synthesized protein was required for amplification. It appears that this amplification is selective for certain of the cRNA's of influenza (Inglis, S.C., Conti, G. and Mahy, B.W.J., reported at Negative Strand Virus Symposium, Cambridge, 1977),resulting in the time-dependent expression of viral proteins (Skehel, 1973). Previous work had suggested that only some of the RNA's were transcribed in early stages (Avery and Dimmock, 1975) and subsequently (Glass et al., 1975) that they were all present but some in greater quantity than others.

(c) Significance of polyA(+) and (-) strands

At least some of the influenza cRNA's are polyadenylated post-transcriptively (Etkind and Krug, 1974; MacNaughton et al., 1975) and although cordycepin (an inhibitor of polyA addition) does not inhibit FPV multiplication in CEF (Mahy et al., 1973), it does in BHK cells, and this is due to the different specificities of host cell enzymes (Rochovansky and Pons, 1975). Glass et al. (1975) first reported that polyA containing cRNA's hybridized to a maximum of around 80% of the genome whilst polyA(-) RNA's were complementary to 95%. Hay et al. (Hay, A.J., Fellner, P, Smith, J., Abraham, G. and Skehel, J.J. at Negative Strand Virus Symposium, Cambridge, 1977) have found that these two populations comprise the viral messenger RNA and progeny template RNA respectively. The polyA(+) strands are formed by premature termination by the transcriptase and are located exclusively on polysomes. They are present in different amounts and could account for the asymmetric distribution of proteins. In contrast, the polyA(-) strands are present in roughly the same amounts. In the presence of cycloheximide, no polyA(-) RNA was observed, and the ratio of polyA(+) strands was 'fixed' at the point at which the inhibitor was added.

(d) Host cell involvement

Minor and Dimmock (1975, 1977) have classified a variety of RNA synthesis inhibitors according to their action on viral protein synthesis. In each group there are at least two drugs (or treatments) of different nature having the same major action. Group II (α-amanitin, daunomycin, nogalomycin) inhibit all viral protein synthesis and allow host protein synthesis to resume. The principal action of these drugs is on the DNA-dependent-RNA polymerase II. Group IV (camptothecin, uv-irradiation) inhibit synthesis of the late proteins M, HA and NA at all effective doses. Camptothecin has been found to inhibit rRNA synthesis preferentially. Group III (actinomycin D, mithramycin, echinomycin) act like IV at a critical dose and like II at higher dosages. Thus it appears that the amplification of transcription of the early RNA's requires a nucleoplasmic RNA synthesis (polymerase II inhibited by α-amanitin) whilst that of the late RNA's, nucleolar RNA synthesis (inhibited by camptothecin).

Using cell fusion to insert a metabolically dormant avian erythrocyte nucleus into cells specifically blocked with actinomycin D prior to infection, Kelly and Dimmock (1974) were able to demonstrate that the avian nucleus was capable of supporting the first stage of virus protein synthesis. NP was detected in the avian nucleus and subsequently in the heterokaryon cytoplasm, but none of the late proteins were ever observed. Minor and Dimmock (1976) showed that heterokaryons formed from enucleated BHK cells and avian erythrocytes were also competent to support this first stage of virus growth .

Cellular DNA-dependent-RNA synthesis, early after infection, had been implicated in influenza virus multiplication (Borland and Mahy, 1968). Two peaks of RNA synthesis of which only the first is inhibited by α-amanitin have been observed by liquid scintillation counting (Mahy et al., 1972) and by cell autoradiography (Armstrong and Barry, 1974, 1975). Recently, the effects of α-amanitin on influenza virus growth in a cell line whose RNA synthesis was resistant to α-amanitin (AMAI) and a wild type CHO cell line have been determined (Spooner and Barry, 1977). It was found that in the presence of α-amanitin the virus grew normally in the mutant cell line whilst it was completely inhibited in the wild type. Thus it appears that host cell DNA-dependent-RNA polymerase II is involved in the early stages of influenza transcription. There may also be other host cell requirements. Rochovansky has reported two cell factors, one of them a pyrophosphatase, which enhance the activity of the virion polymerase (Rochovansky, O.M. and Ciraolo, P.J. at Negative Strand Virus Symposium Cambridge, 1977).

(e) Synthesis of virion(-) RNA

Progeny vRNA might be transcribed from the unpolyadenylated full-length transcripts of the input virion RNA according to the scheme

reported by Hay (see above). From his work it appears that a newly synthesized protein is required in the production of these templates (since their formation is prevented by cycloheximide) although it has been known for some time that the total process of vRNA synthesis was sensitive to cycloheximide (Scholtissek and Rott, 1970; Pons, 1973). This had been postulated to be due to a requirement for new NP to stabilize the newly synthesized RNA or a requirement for a viral or cell protein as a cofactor in the synthesis. Certainly vRNA synthesis seems to be linked to NP synthesis (Scholtissek et al., 1969). Alternatively, since cRNA synthesis is prolonged under these conditions, a protein may be required to effect the switch from cRNA to vRNA.

It has been widely reported that vRNA synthesis is insensitive to actinomycin D, at least over short periods (Rott et al., 1965; Scholtissek and Rott, 1970; Pons, 1973). This is consistent with the limited period of sensitivity of virus multiplication to the drug (Barry et al., 1962; Barry, 1964). However, actinomycin D does cause a reduction in virus production, even when added at later times (Gregoriades, 1970; Scholtissek and Rott, 1970; Skehel, 1973) which suggests that this interference with cRNA synthesis eventually affects vRNA synthesis.

(f) Site of synthesis of virion RNA

The site of vRNA synthesis is not clear at present. Two reports have tentatively suggested a nuclear location. Avery (1974) detected labelled vRNA in the nucleus of infected cells, but pulse lengths of 1 h were necessary to incorporate sufficient counts for analysis. Using base composition as an indicator of (+) or (-) strand RNA, Krug (1971, 1972) found that the viral RNP's in the nucleus were predominantly (-) strand, whilst in the cytoplasm there were populations of both (+) and (-) strand RNA, but he again used 1 h pulse times.

(g) Association of viral RNA with NP

Although Krug (1972) detected free cRNA in the cytoplasms of infected cells, both Blobel (1971) and Pons (1971,1972) were led to conclude that viral RNA was only found in association with NP in a ribonucleoprotein structure. Pons presented evidence that NP was added to cRNA during its synthesis as he was able to locate incompletely synthesized RNA molecules in an RNP fraction.

If cRNA is unstable when not associated with NP, as its absence as a free entity during the virus growth might suggest, it begs the question of how the first messages are transported into the cytoplasm, if indeed they are. The initial transcripts have no newly synthesized NP to protect or stabilize them, and if NP is required they would probably have to sequester pre-existing NP by displacing virion RNA from RNP's.

3C. Influenza Virus Multiplication Cycle: Protein Synthesis

Virus directed protein synthesis occurs very rapidly after influenza virus infection. Lamb and Choppin (1976) could detect newly synthesized viral proteins after only 45 min,and protein synthesis is well under way by the time of maximal cRNA synthesis which is at 2-2.5 hpi in CEF cells (Scholtissek and Rott, 1970; Glass et al., 1975). The maximal synthesis of individual viral proteins may occur at different times, and there are considerable variations, certainly of a quantitative and possibly of a qualitative nature,in different cell systems and with different strains of virus. No clear pattern has yet emerged, and it is prudent to be wary in the extrapolation of the results from a single virus strain-host cell system to that of influenza multiplication in general.

(a) Temporal expression of influenza proteins

Though first apparent in earlier results (Skehel, 1972), temporal expression of FP/Rostock proteins in CEF cells was reported by Skehel (1973). He found that, at 31°, the P proteins, NP and NS1 appeared first, and M, at least, was not detectable until later. This pattern could be 'frozen' by addition of actinomycin D to prevent further mRNA synthesis. A newly synthesized protein was implicated in the 'switch' from early to late proteins due to the delay, after the removal of a cycloheximide block established early in infection, between the expression of early proteins (which occurred immediately) and the expression of late proteins (which occurred 30 min later). This has recently been questioned, however, by more extensive studies (see below). The phenomenon has also been observed in infected CEF and BHK cells at 37° (P.D. Minor, personal communication), but in WSN infected BHK cells Meier-Ewert and Compans (1974) saw only an increase in the amount of M synthesized late in infection.

In WSN infected CEF cells the ratio of M and HA to other proteins synthesized after the release from a cycloheximide block depended on the duration of the inhibition (Lamb and Choppin, 1976). From this they concluded that regulation of protein synthesis occurred in this system. They also described differences in the amplification of viral proteins in this and a WSN/MDBK cell system, and this variation between systems was the theme of their recent report (Lamb, R.A., Choppin, P.W. at Negative Strand Virus Symposium, Cambridge, 1977). For example, in CEF, L and BHK cells, M was detectable from 0.5 hpi, and the rate of synthesis of NP, M and NS1 remained constant to 6 hpi, whilst in CHO-S, HeLa, MDBK and CV-1 cells, the rate of synthesis of M increased throughout this period. On treatment with inhibitors which restricted RNA synthesis to primary transcription different cell lines again gave rise to variations in the expression of viral proteins.

These data could be interpreted to mean that primary transcription of the 'late' viral RNA's requires a host function which involves a protein which is present in variable amounts in cell lines. The inhibition of protein synthesis in cell lines with excess of this protein thus has no effect, whilst in cell lines in which this protein is limiting late viral proteins are not observed at the same time as the early species and their expression is sensitive to the inhibitors. This protein could act at the level of transcription or translation.

(b) The synthesis of viral proteins

Although a large molecular weight precursor of influenza viral proteins was suggested from the work of Etchison et al. (1971), following the discovery of such a mechanism in polio infection, subsequent studies have not substantiated this (Compans et al., 1970; Schulze, 1970; Skehel and Schild, 1971; Skehel, 1972). However, the HA0 polypeptide is a primary gene product from which the HA1 and HA2 molecules are derived by proteolytic cleavage (Lazarowitz et al., 1971; Klenk et al., 1972; Skehel, 1972).

The haemagglutinin of the virus can exist in the uncleaved or cleaved form and this operation is performed by the host cell in some cell lines (Klenk et al., 1972, 1974; Hay, 1974) or by a serum protease after its activation by the host cell (Lazarowitz et al., 1973) and can be performed in vitro (Lazarowitz et al., 1973; Stanley et al., 1973; Klenk et al., 1975). The likelihood of this event occurring depends on both the conformation of the glycoprotein around the cleavage site and the specificity and activity of the host enzyme (Klenk et al., 1977). The haemagglutinin molecules are glycosylated by host cell enzyme systems and a non- or incompletely glycosylated form has been located after blocking glycosylation by glucosamine (Klenk et al., 1974). Inhibition of protein synthesis results in the immediate inhibition of glucosamine incorporation whilst fucose addition continues for 15 min (Stanley et al., 1973). This was interpreted as indicating that the sugar chains contained glucosamine before fucose though the mechanism and significance of this interdependence is unclear.

All the other influenza virus proteins appear to be primary gene products with the possible exception of the non-structural proteins NS2 (approximately 11 k daltons) and other small molecular weight virus induced species sometimes found in infected cells. First observed by Skehel (1972) and subsequently by other workers (Krug and Etkind, 1973; Hay, 1974; Minor and Dimmock, 1975), NS2 may be a degradation product of NS1 along with several other proteins observed by Lamb and Choppin, (Lamb, R.A. and Choppin, P.W. at Negative Strand Virus Symposium, Cambridge, 1977) by two-dimensional tryptic peptide analysis. Matrix protein was found, on treatment with trypsin, to give two specific degradation products of 13 and 6 k daltons (Oxford and Schild, 1976) and these may be analagous to some of those observed in vivo.

3D Location and Movement of Viral Proteins between Compartments of the Host Cell

(a) Techniques

The association of viral proteins with cell compartments detailed in this section rest heavily on the techniques of cell fractionation. The problems and pitfalls of preparing nuclear fractions are dealt with in the Appendix. Cytoplasmic fractionation studies have generally followed the method of Caliguiri / and Tamm (1970) which involves the use of centrifugation on discontinuous sucrose gradients to produce discrete bands of membraneous material. These generally distinguish rough and smooth endoplasmic reticulum, a plasma membrane fraction, a fraction containing free ribosomes and a soluble fraction. The fractions are commonly identified by their appearance by electron microscopy (Compans, 1973; Klenk et al., 1974), and by their enzyme content (Hay, 1974). This is obviously only a crude distinguishing procedure, giving the predominant membrane species in each fraction The problems of cross-contamination of fractions, both by viral protein reassociating with adjacent fractions, and by membrane resedimenting at different densities, has been described (Compans, 1973). Nevertheless, distinct populations of viral protein can be isolated from some of these fractions and, furthermore, they can sometimes be chased from fraction to fraction in the course of virus multiplication. Thus these results give one of few insights into the movement and location of viral polypeptides within the cytoplasm of the infected cell.

Another powerful technique for following the movements of viral components is indirect immunofluorescence. In this method, infected cells are fixed, then exposed to a specific antiserum. This is usually raised in rabbits against the purified viral component, and absorbed extensively against disrupted uninfected cells and unwanted viral components. The use of recombinants has enabled specific antisera to be raised against the surface glycoproteins (Kilbourne et al., 1968). After treatment with specific antiserum, the cells are then exposed to an antiserum to the first antibodies (i.e. anti-rabbit IgG) to which a fluorescent molecule has been attached. After washing, concentrations of viral components can be located by their fluorescence when observed under a microscope with uv optics.

(b) Location of mRNA's as evidence of the initial site of viral protein synthesis

It was thought that the distribution of cRNA segments between membrane bound and free ribosomes would be distinct and non-overlapping and thus hybridization to excess with total vRNA would be incomplete for both populations. This was not found to be the case, although an asymmetric distribution of the cRNA was observed (Glass et al., 1975). The RNA's coding for the glycoproteins and M associated predominantly with membrane bound ribosomes, whilst the P proteins, NP and NS1 were found mainly with free ribosomes (McGeoch et al., 1976).

(c) Nucleoprotein

This protein is present in the nucleocapsid of the virus and can be located in similar structures in the infected cell (see Introduction: 2B: the Polypeptides of the Virus). By radiolabelling and subsequent analysis by polyacrylamide electrophoresis, NP has been shown to be synthesized in the cytoplasm and to migrate rapidly to the nucleus (Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Krug and Etkind, 1973; Hay and Skehel, 1975). There have been no reports of this protein returning to the cytoplasm by this method of detection.

By fluorescence methods the 'g' or ribonucleoprotein antigen found in the virion was located solely in the nucleus of CEF cells at 3 to 5 hpi whilst the cytoplasm exhibited no fluorescence. 4 h later, fluorescence was detectable in the cytoplasm, and by 14 hpi the whole cell was fluorescing, completely obscuring the nucleus (Breitenfeld and Schafer, 1957). These experiments were repeated by Maeno and Kilbourne (1970) who reported a similar result using monospecific antisera to NP in an infected human cell line. It has been reported that an 'emptying' of the nucleus can be observed in FPV infected CEF cells occurring only 1½-2 h after the initial appearance of fluorescence in the nucleus (Fraser, 1967; Kelly and Dimmock, 1974) In contrast to the previous workers who observed only that the cytoplasmic levels of NP rise later in infection until the whole cell fluoresces.

It has been a tantalizing prospect to demonstrate the movement of NP back into the cytoplasm (representing virion RNP's on their way to assembly into virions) but evidence for this is as yet lacking. Hay and Skehel (1975) could find no evidence for any such movement, though much of their observation concerned proteins synthesized late in infection which may not take part in virion formation. These authors also studied the kinetics of accumulation of NP in the nucleus ($t_{\frac{1}{2}} \sim$ 3 min) and its incorporation into virions (time lag 45 min - see later) and decided that nuclear NP could be destined for assembly in virions. In contrast, Krug (1972) decided that RNP's which he isolated from sucrose gradients of the nuclear fraction from infected cells were not the precursors of cytoplasmic RNP's.

Efforts to subfractionate nuclei, to further refine the location of NP, are hampered by the reassociation of nucleoplasmic elements with all fractions and the fragility of nucleoli, especially later in infection. However, both Taylor et al. (1970) and, more recently, Krug and Soeiro (1975) have suggested that NP is predominantly nucleoplasmic though Krug and Etkind (1973) also found substantial amounts in their nucleolar fraction.

Thus there is a general concensus that NP, synthesized in the cytoplasm, migrates rapidly to the nucleus and may be concentrated in the nucleoplasm. There is no evidence for substantial amounts of this protein returning to the cytoplasm. However, the distribution of the NP antigen alters through the course of infection, and there are reports of an 'emptying' of the nuclei.

The location and movement of NP in the cytoplasm has been extensively studied. There is general agreement that NP is initially present in the soluble fraction, but much of it subsequently becomes sedimentable (Compans, 1973; Hay, 1974; Klenk et al., 1974). In its sedimentable form, the NP is found at a density intermediate between rough and smooth endoplasmic reticulum and is probably present as a ribonucleoprotein complex cosedimenting with the membranes. Hay and Skehel (1975) isolated a 40 s complex from this region of the gradient which contained NP and P1 and 2 and exhibited RNA polymerase activity. NP is present in great excess relative to the other proteins compared with this ratio in virions, and is not chaseable in the cytoplasm (Meier-Ewert and Compans, 1974), a common observation (Hay, 1974; Klenk et al., 1974). By pulsing infected cells at various times after infection, chasing to the completion of the virus growth cycle and then analyzing the virions produced, it has been determined that most of the NP found in virions is synthesized early in infections in contrast to M and HA (Meier-Ewert and Compans, 1974 Krug, 1972).

(d) Non-structural protein: NS1

This protein migrates to the nucleus and moves more rapidly than NP (Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Hay and Skehel, 1975).

It is thought to be concentrated in the nucleolus from evidence provided by the subfractionation of nuclei by Krug and Etkind (1973) and most recently with the additional refinement of purification of the nucleoli on Renografin gradients (Krug and Soeiro, 1975). Both of these studies encountered considerable problems from the reassociation of nucleoplasmic components, and the loss of nucleolar associated protein during processing. A more direct approach, using the immuno-fluorescent technique, showed that a non-structural virus specified antigen was concentrated in the nucleolus (Dimmock, 1969).

NS1 is also present in the cytoplasm. After a report by Pons (1972) that NS1 was found in association with ribosomes, much interest was centred on its possible role in redirecting cell protein synthesizing machinery. Compans (1973) found NS1 in the cytoplasm to be exclusively polysomal whilst Klenk et al. (1974) also found it in polysome fractions. However, it was reported (Krug and Etkind, 1973) that although NS1 appeared in association with ribosomes in the normal course of extraction, no associated NS1 was found if the ribosomes were extracted in the presence of high salt, puromycin and magnesium ions, in which state they were purported to be reactivable. This, they suggested, indicated that NS1 was not behaving like a ribosomal structural protein. However, a looser yet specific association cannot be ruled out. In support of this type of specific association, Klenk et al. (1974) found that the association of NS1 with ribosomes was partially dependent on viral glycosylation processes since a glucosamine block reduced the amount of NS1 recovered in the ribosome fractions.

A curious phenomenon has recently been reported in which crystalline aggregates of NS1 were observed very late in influenza infected cells (Morrongiello and Dales, 1977) which may correspond to the aggregates of unknown composition reported by Compans and Dimmock (1969). Apart from NS1 these complexes contain traces of a helical component containing NP, possibly the remains of a replication complex, and are fringed with ribosomes. The relevance of this structure to the influenza virus growth cycle is unknown, but the phenomenon may enable the preparation of NS1 for X-ray diffraction study and sequencing.

(e) The P proteins

Until recently (Inglis et al., 1976; Lamb and Choppin, 1976), these proteins have not been readily detected due to the small amounts present in the infected cell. This is particularly galling considering their probable participation in the processes of RNA synthesis of influenza. Although Krug and Etkind (1973) detected P proteins in nucleoli and nucleoplasm, Hay and Skehel (1975) found them barely detectable in the nucleus and predominantly in the soluble cytoplasm. This was also reported by Compans (1973) and Klenk et al. (1974) found 'large amounts' of the P proteins in this fraction. The P proteins seemed to follow NP with regard to their kinetics of incorporation into virions (Meier-Ewert and Compans, 1974; Hay and Skehel, 1975).

(f) The glycoproteins, HA and NA

Most workers have found that of the glycoproteins it is easier to follow the path of the haemagglutinin molecule through the infected cell than that of the NA which is renowned for its poor labelling properties. HA appears to be synthesized on rough ER and most of the HA is located in this fraction when glycosylation is inhibited by glucosamine (Klenk et al., 1974; Hay, 1974). The molecule can be chased into smooth ER and concentrates here (Compans, 1973), and in the plasma membranes (Hay, 1974; Lazarowitz et al., 1971; Compans, 1973) where it becomes predominant later in infection. The site of cleavage appears to depend on the host cell and the configuration of the glycoprotein as detailed earlier, but cleavage has been observed in fractions of smooth ER and of plasma membrane. By using an ingenious technique involving the isolation of plasma membrane fragments and their separation on the basis of precipitation by red blood cells, Hay (1974) separated populations of fragments with and without haemagglutinating activity. Using this method, he was able to demonstrate that HA molecules were inserted in the cell membrane at positions distinct from the site of virus budding. These proteins could be chased into the sites of maturation where cleavage occurred, and thus it appeared that it was the cleaved HA's which specified the site of virus assembly.

Neuraminidase moves more slowly from its site of synthesis on rough ER through smooth ER to plasma membrane than the haemagglutinin (Hay, 1974) and becomes more generally distributed in the plasma membrane than HA1 and HA2 which are localized in discrete regions. It has been postulated that a difference in glycosylation rates between the two glycoproteins accounts for this difference in incorporation kinetics, but firm evidence is lacking (Hay, 1974).

The visualization of HA and NA by immunofluorescence has indicated that both glycoproteins are concentrated initially at sites peripheral to the nucleus (Breitenfeld and Schafer, 1957; Maeno and Kilbourne, 1970; Kelly and Dimmock, 1974). Sometimes the fluorescence around the nucleus is asymmetric with concentrations at the poles of the cell (Maeno and Kilbourne, 1970). Later in infection, the fluorescence is predominantly on the cell margin and the level in the cell cytoplasm is raised.

The movements of the antigens correlate well with the results from cell fractionation and presumably reflect the synthesis and processing route of these components and their site of assembly at the cell membrane.

(g) The matrix protein

Recently antisera has been prepared against the matrix protein and, using the indirect immunofluorescent technique, Oxford and Schild (1975) found that in the majority of cells M remained cytoplasmic but that, in about 5% of them, nuclear fluorescence was observed. Gregoriades (1973) reported M in the nucleus, using an acidified chloroform-methanol extraction which was claimed to remove only this viral protein. However, recently Gregoriades has found, using tryptic peptide analysis, that both M and NS1 are extracted (Gregoriades, A. at Negative Strand Virus Symposium, Cambridge, 1977).

Hay and Skehel (1975) found that M could be chased into the nuclear fraction but at a slower rate than that of NP or NS1. From the kinetics of its incorporation into virions, these authors concluded that nuclear M cannot be destined to move subsequently to the cell membrane to be assembled into virions. These workers studied proteins synthesized late in virus multiplication (5 hpi in CEF) which might not get incorporated into virions. Given the very large amounts of M synthesized at this time, it is doubtful whether movements of a small amount of M from the nucleus would be observed.

There is general agreement that, using the cytoplasmic fractionation techniques described, M protein cannot be located in subviral particles prior to their incorporation into virions (Compans, 1973; Hay, 1974). This protein can be chased from rough ER to the smooth membrane fraction, but it accumulates here and becomes the predominant viral protein (Klenk et al., 1974) and cannot be chased (Compans, 1973). Both Lazarowitz (1971) and Hay (1974; Hay and Skehel, 1975) found M in the plasma membranes of infected cells. Some part of the smooth endoplasmic reticulum fraction of Klenk et al. (1974) and Compans (1973) is probably plasma membrane; thus the large amount of matrix protein found there is in accord with the observations of Lazarowitz and Hay. This idea is reinforced by the apparent binding of added virus to this fraction, since virus binds to plasma membrane. The result of this is a change in the density of the virus band on sucrose gradients (Klenk et al., 1974).

Meier-Ewert and Compans (1974) found that matrix protein could bind to membranes non-specifically after fractionation and might have an affinity for them, thus indicating caution in the interpretation of fractionation studies. They suggested that M is incorporated into virions by a different pathway from both the glycoproteins and the nucleocapsid. This is supported by the data of Hay (1974) who found that M was incorporated directly after synthesis into virions, whereas HA was incorporated 25 min following synthesis, and NP 45 min after synthesis. The arrival of M, Hay suggested, was thus the final prebudding event which might trigger virus budding. It had been reported previously that synthesis of M was rate-limiting in virus production (Lazarowitz et al., 1971; Stanley et al., 1973) and this would agree with the data of Hay. However, his study also showed that matrix protein was incorporated linearly for some time after its synthesis which, together with its inability to be chased, led him to postulate that M protein was incorporated into virions from a large pool in the infected cell and thus was not a rate-limiting step (Hay, 1974).

(h) Summary

After synthesis has occurred in the cytoplasm, the evidence indicates that influenza virus protein transport may involve a number

of separate pathways. NP, M and NS1 migrate to the nucleus (M possibly more slowly than the other two) where NS1 becomes associated with the nucleoli whilst NP remains nucleoplasmic. There is little convincing evidence in the literature to date for a move by any of these proteins back into the cytoplasm, although this is assumed to occur in the accepted dogma of influenza multiplication. In the cytoplasm, the putative glycoproteins move from their site of synthesis, on rough ER close to the nucleus, via the smooth ER to the plasma membrane, undergoing glycosylation (HA and NA) and cleavage (HA) en route. They are incorporated in the plasma membrane, probably by normal cell processes, and then haemagglutinin is rapidly concentrated into localized areas. The nucleocapsid is incorporated after a 45 min delay which may involve it migrating to and from the nucleus. Another transport pathway incorporates the matrix protein into virions immediately after its synthesis, possibly triggering the budding of the virion on arrival at the plasma membrane. The P proteins are assumed to mirror NP in their movements though evidence is lacking. NS1 in the cytoplasm is associated predominantly with polysomes and ribosomes, which might, together with its affinity for the nucleolus, suggest a function in modification or direction of the host cell protein synthesizing machinery.

4. Movement of the Proteins of Adenovirus between Cytoplasm and Nucleus and the Suggestion of a Mechanism for such Transport

Adenovirus is a DNA virus (Class I, Baltimore, 1971) and differs in many respects from influenza, not least in respect of virion assembly in the host cell nucleus, yet they have important nuclear processes in common. The genetic material is assembled and packaged into cores in the nucleus, and from that site appear to come the signals which dictate amplification or switching of virus synthetic functions.

Adenovirus

Adenovirus is a double-stranded DNA virus with a linear genome of 23×10^6 daltons capable of coding for 1.1×10^6 daltons of proteins or about 20-30 species. It is a non-enveloped virus with an icosahedral shell of protein subunits, the capsid structure, with projecting spikes at each vertex and an inner core containing protein and DNA. The major unit of the capsid is the hexon (protein II) of 120 k daltons; the spike consists of the penton base (III) and fibre (IV). A number of proteins are associated with the capsid (VI, IX amongst others). The inner core is composed of the DNA and a core protein VII , and an associated minor species V (Philipson et al., 1975). The assembly of progeny virions takes place in the nucleus.

Adenovirus: Passage of the Input Virion to the Nucleus

There are three stages of uncoating of the input virus recorded in the literature. The first, which removes the penton capsomers, may take place alongside the pores in the nuclear membrane whilst in the second stage the DNA-protein core enters the nucleus leaving the capsid structure outside (Chardonnet and Dales, 1972). Finally, within the first 2 h of infection, further proteins are removed from the core to leave a deproteinized DNA molecule (Lonberg-Holm and Philipson, 1969). It has been suggested that microtubules may be involved in the transport of adenovirus from the plasma membrane to the nucleus since the virus is often found close to them when observed by electron microsocopy. A specific association between these organelles and the virus has been reported (Luftig and Weihinger, 1975) and this correlates with the slowing of virus transport to the nucleus, when these cells were treated with vinblastin, a drug which disorganizes microtubular structure. This has been further investigated (Weatherbee et al., 1977) when it was found that a protein of high molecular weight associated with the microtubules was required for specific virus binding. However, the amount of non-specific binding was high, and in these studies it is difficult to prove that such associations are relevant to the transport process. Nevertheless, this is one of very few investigations of the actual mechanism of such transport processes and provides an indication of the sort of mechanism which may move influenza proteins between cytoplasm and nucleus.

Adenovirus: Timescale of Macromolecular Synthesis

'Early' proteins are synthesized first and precede DNA synthesis, while 'late' proteins are synthesized as genome replication proceeds. Early proteins comprise near to 50% of the genome and are detectable at between 2 and 6 hpi. At least one viral antigen (T) is expressed prior to DNA synthesis, and probably also a second (P). DNA replication commences at 6 to 8 hpi and at this point late messages appear. These code for the structural proteins of the capsid which appear to be assembled into an empty structure into which the DNA-protein core is inserted (Horwitz et al., 1969; Sundquist et al., 1973). The first virions can be detected at 10 to 11 hpi and maximum release occurs at 20 to 24 hpi.

Adenovirus: Movement of Proteins within the Infected Cell

Velicer and Ginsberg (1970) when analysing late viral proteins on sucrose gradients found that polypeptide chains were released from polysomes within 1 min of chase. Within 6 min their transport into the nucleus was complete. This coincided with their assembly into multimeric capsid proteins, and the development of antigenicity. Horwitz et al. (1969) also found the rapid development of capsomeres after protein synthesis. It is thought that this assembly occurs within the nucleus and thus transport into that organelle must be very rapid. The core protein precursor P-VII is found in the nucleus where its cleavage to VII is very slow (50% in 12 h) (Anderson et al., 1973).

A survey of ts-mutants (Russell et al., 1972) produced two groups which were defective in capsid protein. One group failed to make the hexon antigen altogether, whilst in the other group the hexon antigen was detected by immunofluoroescence, but it accumulated outside the nucleus. It was presumed that either the protein was defective such that it was not able to enter the nucleus (whilst still retaining its antigenic configuration) or that some other viral component was involved in its transport through the nuclear membrane and this was absent or non-functional. Arginine deprivation allows the synthesis of structural proteins, but again no migration to the nucleus can be detected (Mark

and Kaplan, 1971). Since the synthesis of these proteins is little affected, it suggests that the transport is effected by another newly synthesized, virus-induced protein which is rich in arginine.

The synthesis and accumulation of the early, DNA banding protein (DBP) of molecular weight 70-75 k daltons has been followed by indirect immunofluorescence techniques (Sugawara et al., 1977). DBP accumulates in the cytoplasm from 3-7 hpi and appears as a diffuse area of stain. Concentrated 'dots' of fluorescence appear in the nucleus from 8-14 hpi and the cytoplasmic levels are drastically reduced. A link between the synthesis of DBP and DNA replication was suggested since DNA synthesis begins at 7 hpi and in the presence of cytosine arabinoside (an inhibitor of DNA synthesis) DBP continues to accumulate in the nucleus.

These then are some tantalizing indications of the specificity and mechanism of virus protein transport and suggest that it may be possible to probe the control of influenza protein transport within the infected cell by the use of selective inhibition or temperature sensitive mutants of transport processes.

5. Influenza Virus Release

In contrast to the controversy over virus entry into the cell, there is general agreement that influenza virus is released by budding from the plasma membrane. The cellular origin of the viral membrane and the sequence of assembly of viral components has already been described. Electron microscopy has shown virus budding from the membrane (Compans and Dimmock, 1969) and although newly synthesized virus is also seen in what are apparently cytoplasmic vacuoles, these have been shown by thorotrast staining to be derived from the plasma membrane.

Influenza virus release: the assortment of RNA segments

With the knowledge that the genome is composed of separate RNA segments, the question of their sorting and packaging in the virion arises. Although there may be precise selection of the correct set of segments, the low infectivity of some strains of influenza has led some investigators to suggest that random or near random packaging of eight segments may occur (Hirst, 1962, 1973). However, this would lead to very low PFU:particle ratios.

It is possible that extra segments may be incorporated (Compans et al., p. 99-100, 1970b) or there may be certain restrictions on the number of segments of each size class incorporated. For example, it has been calculated that a random incorporation of three large segments, three intermediate and two small segments would lead to a PFU-particle ratio in the range similar to that observed as would the incorporation of one extra segment in an entirely random arrangement (P.D. Minor, personal communication). The observation that aggregation of virus particles enhances infectivity suggests that defective particles can complement one another, especially if they enter the cell at the same point (Hirst and Pons, 1973). This might be suggestive of random packing by indicating one mechanism by which the virus may have overcome its disadvantages. It would not be relevant here to review in any greater depth the investigations of this aspect of influenza virus. However, the possibility of an essential multi-particle viability would necessitate a fascinating change of viewpoint from which to base a teleological consideration of the virus growth cycle.

6. The Nuclear Role in Influenza Virus Infection

The particular nature of involvement of the nucleus of the host cell in infleunza virus multiplication is unique amongst viruses. The intention of this short survey is to bring together the data, some of which has already been presented in earlier sections, and present it to highlight our present knowledge of the nuclear events. The first suggestion that host nuclear functions were involved came when Barry and coworkers (1962; Barry, 1964) demonstrated the inhibition of influenza multiplication after treatment of host cells with uv light or actinomycin D. Clinching evidence of this nuclear requirement came in 1974 when Follet et al. demonstrated that influenza did not multiply in enucleate cells in conditions which supported the growth of paramyxoviruses.

The paradox in nuclear involvement had been apparent since it was demonstrated that neither the in vitro virion RNA transcriptase activity nor the cell free synthesis of virion proteins directed by viral mRNA extracted from infected cells were inhibited by AMD. Recently Content et al. (1977) have shown that virion proteins can be synthesized in a coupled transciption/translation system from disrupted virions, and this coupled process too is resistant to AMD. Thus it appears that much of the virus multiplication cycle can be created in vitro without the requirement of nuclei.

Furthermore, the only RNA step yet to be accomplished in a cell free system, the replication of vRNA from cRNA, appears to be insensitive to actinomycin D *in vivo*, and thus might not involve host nuclear processes.

Substantial evidence has now accumulated that the host DNA-dependent-RNA polymerase II (pol II) is involved in influenza virus replication, though the precise nature of its role is still unclear. Whether this activity is the whole or only part of the host nuclear involvement is not, as yet, resolved. Borland and Mahy (1968) described a DNA-dependent-RNA polymerase activity induced in the infected cell. Later, following the demonstration (Rott and Scholtissek, 1970) that influenza RNA synthesis is sensitive to α-amanitin, a drug which inhibits pol II specifically (Stirpe and Fiume, 1967), Mahy *et al*. (1972) showed that of two peaks of RNA synthesis in infected cells, only the first was α-amanitin sensitive. This observation was subsequently reinforced by cell autoradiography (Armstrong and Barry, 1974) which also showed that this synthesis occurred in the cell nucleus. Recently it has been shown that in cells in which pol II is resistant to the drug, α-amanitin has no effect on viral replication (Spooner and Barry, 1977). The comprehensive study of inhibitors by Minor and Dimmock (1975, 1977) showed that a group of drugs inhibited influenza replication like α-amanitin, but they also described a specific inhibition of the synthesis of some viral RNA's with another group of treatments. Some drugs (including actinomycin D) inhibited the synthesis of some viral RNA's at a critical dose, and all RNA synthesis at higher doses.

The demonstration of early and the absence of late influenza antigens in erythrocyte-actinomycin D treated BHK cell heterokaryons (Kelly and Dimmock, 1974) and erythrocyte-enucleate BHK cell heterokaryons (Minor and Dimmock, 1976) also suggested two host-dependent events, of which only the former was provided by the erythrocyte nuclei. However, it is possible that there is a requirement for a host nuclear process and/or product which is different in intensity between the eight viral segments. This could account for the erythrocyte data and would also accommodate the results of Lamb and Choppin (Lamb and Choppin, 1976; Lamb, R.A. and Choppin, P.W. at Negative Strand Virus Symposium, Cambridge, 1977) who found that all the viral messages were transcribed in some cell systems whereas only some were

transcribed in others. In this case, a host product might be present in limiting amounts, allowing the transcription of the less sensitive viral RNA's but precluding the processing of the RNA's with a greater requirement for the host product. The step involved might well be an initiation of transcript which has not been reliably observed in the in vitro systems, i.e. all in vitro RNA and protein synthesis takes place in the absence of re-initiation of transcription.

The virion polymerase is stimulated in vitro by dinucleotides up to 100-fold (McGeoch and Kitron, 1975) and the role of these as primers in the synthesis of cRNA's has been suggested to mimic a postulated in vivo system in which an RNA primer is synthesized by pol II in the nuclei of infected cells (Plotch and Krug, 1977; Krug, R.M., Plotch, S.J. and Tomasz, J. at Negative Strand Virus Symposium, Cambridge, 1977). There is, as yet, no evidence for this postulated system in vivo.

There is a wealth of purely circumstantial evidence for nuclear involvement, some of which indicates processes that might be sited in the nucleus. There is evidence of transport to the nucleus of the input RNA, input RNP's (though some at least migrate back to the cytoplasm at an early stage in infection), newly synthesized P proteins, NP, NS1 and M and newly synthesized vRNP's.

The location of Influenza RNA synthesis is still controversial. Early reports located virus directed RNA synthesis in infected cells in microsomal fractions (Ho and Walters, 1966; Scholtissek and Rott, 1969; Scholtissek, 1969; Mahy and Bromley, 1970) with a maximum activity at 6 hpi. This is probably newly synthesized polymerase on its way to incorporation in the budding virions. Avery (1974) concluded that since newly synthesized vRNA was found in the nucleus and cRNA in the cytoplasm immediately after a 1 h pulse, that these represented their sites of synthesis.

An RNA-dependent-RNA polymerase has been reported in the nuclei of infected chick cells which rises to a maximum activity at 3 hpi and subsequently declines (Hastie and Mahy, 1973). It is indistinguishable in requirements from the microsomal activity, but the product is only 40% hybridized by vRNA whilst 93% of the cytoplasmic product is

hybridized. Cell autoradiography showed grains predominantly over the nuclei at 3 hpi, the time of maximum RNA-dependent-RNA synthesis, and a cytoplasmic activity could only be detected later in infection and under artificial conditions (Armstrong and Barry, 1974). Stephenson and Dimmock (1975) observed incorporation of labelled uridine into TCA-precipitable counts in both nucleus and cytoplasm shortly after infection. Thus it appears that some cRNA and probably all of the vRNA synthesis occurs in the nucleus.

Materials

1. Radiolabelled Components

These were obtained from the Radiochemical Centre, Amersham, Bucks. and had the following specific activities.

[2-^{3}H] adenosine 5'monophosphate, 15-22 Ci/mmol; [8-^{3}H] - adenosine 3', 5'cyclic phosphate, 20-30 Ci/mmol; [α-^{32}P] adenosine 5'-triphosphate, 0.5-10 Ci/mmol; [^{35}S] - L methionine, 300-800 Ci/mmol; [methyl-^{3}H] thymidine, 18-25 Ci/mmol; [5-^{3}H] uridine, 25-30 Ci/mmol.

2. Electrophoresis Components

Acrylamide was obtained from Fluka AG, Buchs, Germany and B.D.H. Chemicals, Poole, Dorset. N-N'-methylene bisacrylamide and N, N, N', N', -tetramethylenediamine (TEMED) were obtained from Kodak Ltd., London.

3. Chemicals

Analytical grade 2-(3-acetamido-5-N-methylacetamido-2, 4, 6-triiodobenzamido)-2-deoxy-D-glucose (Metrizamide) was purchased from Nyegaard and Co., A/S, Oslo; Nonidet P40 (NP40) from B.D.H. Chemicals, Poole, Dorset; Dowex AG 50 WX4, H-form from BioRad Laboratories, Richmond, California; sheep-anti-rabbit fluorescent immunoglobin from Wellcome Laboratories, Beckenham, Kent. All other chemicals were of the purest grade obtainable.

4. Tissue Culture Media

These were obtained from Flow Laboratories, Irvine, Ayrshire and from Gibco Biocult, London, and prepared according to the Flow Manual (1974, 1977).

GM4 was Glasgow modification of Minimal Eagles Medium with glutamine, non-essential amino acids and crystamycin as specified. It was made 4% in newborn calf serum.

GM0 was as above with no calf serum.

GM4H was as GM4 but without $NaHCO_3$ and with 20 mM HEPES, pH adjusted with NaOH.

199 was as specified with 2% calf serum.

EDC4 was Earles salt solution, pH adjusted with bicarbonate and made 4% in dialysed calf serum.

Methods

1. Cells

Preparation of CEF cells

Primary CEF cells were prepared as described by Morser et al. (1973). These were seeded at concentrations ranging from 5×10^5 to 3×10^6 cells/ml and grown in 5 or 15 cm plastic petri dishes until confluent. Cells were used when confluent.

In some experiments, to avoid the variation in virus uptake encountered in different batches of primary CEF cells, batches with uptake efficiencies of 40-50% were frozen at -70^o and grown up when required. The following procedure was used. 20 ml of CEF cell suspension at a concentration of 60×10^6/ml were made 10% in newborn calf serum and 10% in DMSO. This suspension was dispensed in 2 ml ampoules and frozen in a Union Carbide BF6 biological freezer (LR33-10) to -70^o in 90 min, using liquid nitrogen.

When required, ampoules were thawed out rapidly at 37^o and seeded at double concentration in 199 + 10% calf serum. This was changed for GMEM + 4% c/s after 6 to 10 h.

2. Virus: Stocks

A/FPV/Rostock/34 (Hav1. Nav1) (referred to in future as FP/R) was grown by innoculating 11 day old embryonated chickens' eggs with 10^4 pfu per egg and harvesting the allantoic fluid after 18-20 h growth at 37^o. Allantoic fluid was clarified by centrifugation at 1000 g for 5 min at 4^o. Virus stocks were snap-frozen at -70^o.

The recombinant FP/BEL (Hav. 1. N1) was formed from A/FPV/Dutch/27 (Hav1. Neq1) and A/BEL/42(H0. N1) and was plaque purified 3 times and grown as described above.

Growth Parameters

The infectivity of FP/R was between $1\text{-}3 \times 10^9$ PFU/ml and the PFU:HA ratio 10^6. The infectivity of FP/BEL was 1×10^8 PFU/ml.

Measurement of Viral Activities

Haemagglutination was measured with 0.5% suspension of day old chick red blood cells (Kelly and Dimmock, 1974). Infectivity was measured by plaque assay on CEF cells (Dimmock and Watson, 1969).

Neuraminidase activity was measured using fetuin as substrate by the method described by Webster and Laver (1969).

Preparation of Labelled Virus

The preparation in de-embryonated eggs and purification of [^{35}S] -methionine labelled virus are as described by Dimmock et al. (1977). The preparation of [^{3}H] -uridine labelled virus roller bottle culture is described by Hudson et al. (in press) whilst its purification was as above.

Infection of Cells

Monolayers of CEF cells were washed once with PBS Ca/Mg and infected with 0.1 ml of virus in allantoic fluid at moi = 10-100 (as specified) for 15 min at 20°. Excess virus was washed off with PBS Ca/Mg and the cells washed, then overlaid with prewarmed medium.

3. Proteins

A. TCA Preparation

(a) Cold TCA precipitation. 10 µl samples were diluted into 2 ml distilled water containing 200 µg BSA. 100% TCA was added to 10% final concentration and samples were left for 10 min at 4°. Each sample was then filtered through Whatman GF/C glass fibre filter papers. The filter was washed twice with 4 ml 10% TCA followed by 5 ml of 50% ethanol:50% ether. Filters were dried for 6 h at 37° and counted in triton-toluene scintillant (PPO 0.6% (w/v); POPOP 0.073% (w/v); triton X 100 33% (v/v)).

(b) Hot TCA precipitation. Samples were prepared as above but after the addition of TCA to 10% the samples were heated to 80° for 30 min to hydrolyze charged t-RNAs. They were then cooled to 4° and processed as above.

(c) 'In situ' TCA precipitation. This was performed on cell monolayers in petri dishes to yield both TCA soluble and insoluble radioactivity according to Minor and Dimmock (1977).

B. Ethanol Precipitation.

This procedure was modified from Clewley (1976). 100 µg of cytochrome c was added to the sample and mixed. 4 to 6 volumes of

absolute ethanol at -20° were added, vortexed and held at -20° for at least 30 min. The precipitate was spun for 20 sec in a microcentrifuge and the supernatant poured off. The pellet was dried (a) under a stream of nitrogen, (b) by evacuating over dessicant, (c) at 37° for 3 h. Pellets were resuspended in C_1 with 1.4% SDS, 1.4% β-mercaptoethanol and 0.9 M urea by brief sonication followed by boiling for 5 min.

C. Polyacrylamide gel systems

(a) The phosphate system

This system was as described by Minor and Dimmock (1975) except that slab gels were electrophoresed at 80 V (60-70 mA) for 12-15 h or at 195 mA constant current for 4½ h with forced air cooling.

(b) Tris-glycine exponential gradient gel system

An exponential gradient from 7.5 to 17% acryalamide was produced by mixing the following:

A	B
7.5% acrylamide	17%
0.2% bis-acrylamide	0.01%
0.5 M tris	0.5
0.1% SDS	0.1
0.02% TEMED	0.02
0.02% ammonium persulphate	0.02
50 ml pH 8.6	40% glycerol
	30 ml pH 8.6

in a constant volume system

The stacking gel was as described in Dimmock et al. (1977) as was the electrophoresis buffer. Gels were electrophoresed for 15 h at 100 V constant voltage.

(c) Sample preparation

Samples were denatured with 1.4% SDS, 1.4% β-mercaptoethanol and 0.9 M urea by heating at 95° C for 5 min.

(d) Processing the gel

After electrophoresis gels were fixed by immersion in 25% methanol, 10% acetic acid for 24 h and either fluorographed (see below) or dried

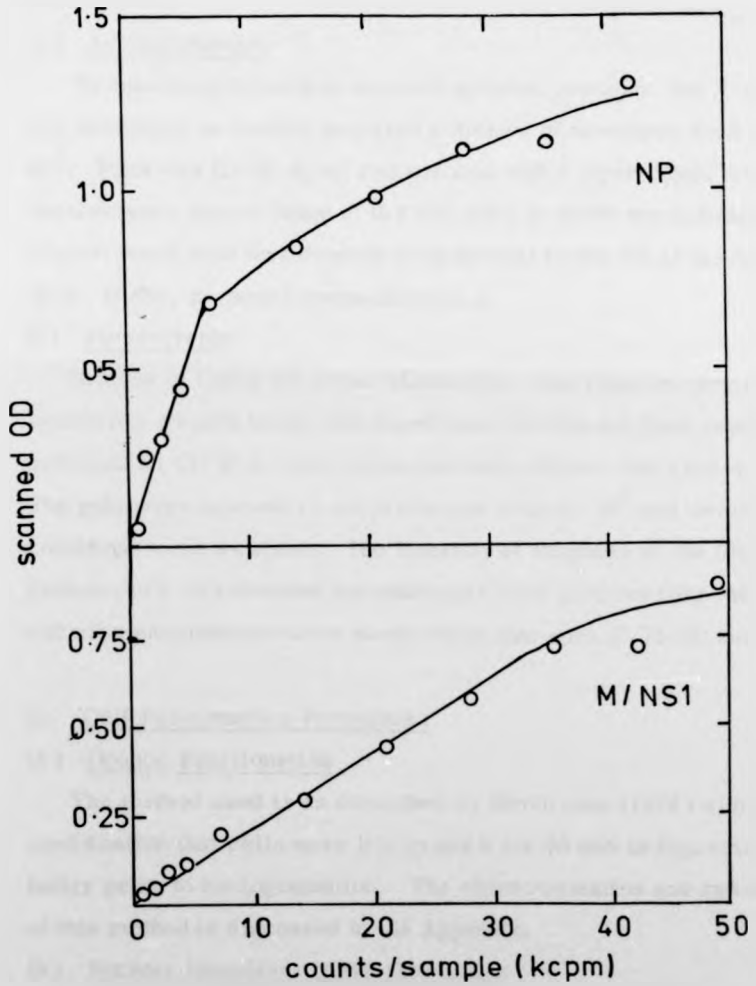

Fig. M/1 The relationship between radioactivity and scanned peak heights for some influenza virus proteins on PAGE. Samples of [^{35}S]-methionine labelled virus proteins containing increasing amounts of radioactivity were analyzed on a phosphate gel. The gel was processed for fluorography on RP Royal XOmat and exposed for 12 h.

down under vacuum supported by 3 MM paper between sheets of silicone rubber in a water bath at 80-90° for 1 h.

Gels were processed for fluorography by the method of Laskey and Mills (1975).

(e) Autoradiography

To ensure unifor nity in autoradiographic intensity, the X-ray film was developed in freshly prepared solutions of developer for 5 min at 20°. Film was fixed, dried and scanned with a Joyce-Loebl micro-densitometer over a range of 0.8 OD units in which the radioactivity of each virus band was directly proportional to the OD of the band (P. D. Minor, personal communication).

(f) Fluorography

Batches of Kodak RP Royal XOmat film were found to vary in their sensitivity so each batch was tested such that the preflash conditions produced an OD of 0.2 (as recommended by Bonner and Laskey, 1974). The gels were exposed to the preflashed film at -70° and development conditions were as above. The linearity of response of the film to radioactivity was checked for individual viral proetins (Fig. M/1) and all measurements were made within that span (0. 75 OD units).

D. Cell Fractionation Procedures

(a) Dounce Fractionation

The method used is as described by Stephenson (1974) with the modification that cells were left to swell for 10 min in hypertonic buffer prior to homogenization. The characterization and rationale of this method is discussed in the Appendix.

(b) Nuclear Monolayer (NML) Formation

This was performed as described by Hudson and Dimmock (1977) and is also discussed in the Appendix.

(c) Preparation of RNP Enriched and Chromatin Fractions.

These were prepared from nuclear monolayers as described by Hudson et al. (in press). Briefly, the nuclear monolayer was scraped into C_5 (140 mM NaCl; 1 mM $MgCl_2$; 10 mM tris-HCl, pH 7. 4) and vortexed for 60 sec. After 30 sec spin in a microcentrifuge, the

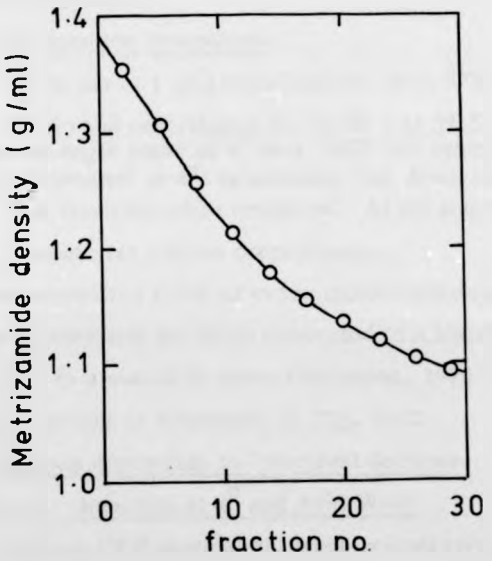

Fig. M/2 Typical density profile of Metrizamide gradient

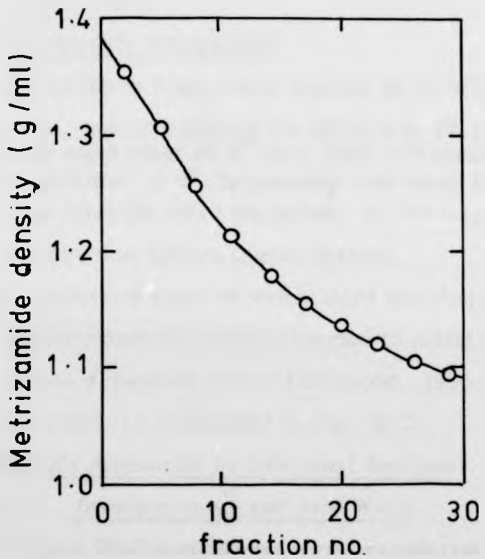

Fig. M/2 Typical density profile of Metrizamide gradient

supernatant was decanted. C_5 was added and this process repeated. The pooled supernatants constituted the RNP enriched fraction. The final pellet was resuspended in 1 mM EDTA, pH 8, and sheared by 10-fold passage through a 26 G syringe needle. This comprised the chromatin fraction.

(d) Metrizamide Procedures

Samples (up to 1 ml) were layered on to 37% (w/v) Metrizamide in C_5 (8 ml) and centrifuged for 62-68 h at 37.5 krpm in a 10 x 10 aluminium angle rotor at 4° on a MSE 165 centrifuge. Gradients were fractionated at 4° by pumping out from the bottom of the tube. 120-150 µl fractions were collected. At all stages precautions were taken to minimise RNAse contamination.

The refractive index of every third fraction was determined by refractometer and the value converted to a Metrizamide density by reference to a standard curve (Rickwood, 1976). The profile of a typical gradient is illustrated in Fig. M/2.

4. Methods Applicable to Individual Sections

Section 1. Infection at 4° and Acid Wash

Confluent CEF monolayers were transferred to 4° and overlaid with cold GMEM for 10 min. The cells were washed once with PBS Ca/Mg (containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$), then infected with 0.1 ml of FP/BEL (moi = 20). After 30 min, when attachment was complete, cells were overlaid with 1 ml PBS Ca/Mg and left a further 60 min. This solution was removed and formed the 'wash'. The cells were then treated with PBS titrated to pH 3 with HCl (acid PBS) for 1 min before being treated with a predetermined equivalent amount of NaOH in PBS to return the solution to neutrality. This solution formed the 'Acid Wash'. Cells were washed once with PBS Ca/Mg and fractionated or overlaid with GMEM prewarmed to 37° for further incubation.

Section 2. A. Radiolabelling Procedures

(a) Starvation 10 min prior to the pulse time, medium was removed from the cells and replaced with prewarmed EDC4 and incubated.

(b) Pulse procedure [^{35}S]-methionine, at concentrations from 10 to 100 µCi, in 250 µl EDC4 was added to the culture after aspirating the starvation medium and the culture incubated generally for 10 min.

(c) Chase procedure The radiolabelled solution was removed and the culture washed rapidly twice with prewarmed GM4 or GM0 with added methionine and then incubated with this medium for the appropriate time. Validity of this procedure is discussed in Section 2.

(d) Salt shock procedure The cells were starved as above but the medium incorporated 150 mM NaCl for 31° experiments and 200 mM NaCl for 37° experiments.

(e) Normalization Fluorographs for the experiments of Fig. 2/14, 17, 18 were normalized in the following way. The OD values for NP in all the fractions of the 1 h chase timepoint were totalled and the total of NP for each of the other timepoints was altered by a factor to bring them to the same value. This factor was then used to adjust all the protein values in each of the fractions at that timepoint. Variations of 10-30% were observed but it appeared that they were completely random. In Fig. 2/14 the values are displayed as a proportion of the total present at each timepoint since at short chase times incorporation into protein is still occurring.

B. Immunofluoresence Procedure

(a) Preparation of antisera Antisera were prepared as described by Kelly and Dimmock (1974).

(b) Preparation of coverslips CEF cells were grown on ethanol washed, heat or u/v sterilized 16 mm glass coverslips in 5 cm plastic petri dishes. Plates were seeded at 1-2 x 10^6 cells per plate and allowed to grow to 30-50% confluency in GM4 medium.

Coverslips were washed once with PBS Ca/Mg and sucked almost completely dry. They were infected with 0.1 ml of suitably diluted virus in allantoic fluid and left for 15 min at 20°. They were washed once with prewarmed GM4 and incubated at 31 or 37°. At appropriate times the coverslips were washed three times with PBS Ca/Mg and dried in a stream of air overnight. They were fixed with acetone for 10-15 min and stored at -20°.

(c) Indirect immunofluorescence procedure Coverslips of fixed cells were spotted in the centre with 10 µl of specific antiserum.

They were incubated for 30 min at 37°In moist conditions and then washed twice in PBS Ca/Mg and dried. Sheep-anti-rabbit fluorescent antibody solution was spotted (10 µl) on to the centres of the coverslips and this was again incubated for 30 min at 37°. After two further washes in PBS the coverslips were removed from their dishes, washed in distilled water and mounted in 90% (v/v) glycerol/PBS. Stained cells were examined on a Reichert Zetoplan Binolux 3 fluorescence microscope and photographed with 15 sec to 4 min exposures on Kodak TriX film.

C. Fractionation of Tissue Culture Fluids

500 µl of sample containing 40 µg of purified virus was loaded on to an 11 ml gradient of 15 to 45% (w/v) sucrose in 0.15 M NaCl, 0.01 M-tris pH 7.4 and centrifuged in an MSE 6 x 14 swing-out rotor at 22,500 revs/min for 120 min at 4°. 500 µl samples were collected from the bottom of the tube, precipitated with ethanol and counted or analyzed on PAGE.

Section 3. Virus Disruption

Virus was disrupted according to Hudson et al. (in press) by treatment with 10% NP40 for 30 min at 20°; according to a method modified from Almeida and Brand (1975) by exposure to 1% NP40 for 5 min at 4°; and by the method of Pons (1971) with a cocktail of 1% NP40 and 0.5% DOC for 4 min at 37°.

Appendix

(a) Enzyme Assays

5'nucleotidase was assayed according to Avruch and Wallach (1971); NADPH-cytochrome c reductase by the method of Phillips and Langdon (1962) using a Unicam SP1800 spectrophotometer with temperature regulated cell holder and automatic sampling, every 60 seconds, of four assays by SP1805 Programme Controller. Adenylate cyclase was assayed according to Solomon et al. (1974) with modifications described in the text. DNA was assayed by the method of Burton (1956) and SDH activity according to Porteous and Clark (1965).

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(b) SFV Experiment

SFV, a kind gift of Dr. S.I.T. Kennedy, was used to infect cells in the following way. A 14 cm plastic petri seeded with 6×10^7 cells was washed and exposed to SFV (moi = 50) in 199 + 2% calf serum with 1 μg/ml AMD. After 60 min at 37° the excess virus was washed out and the cells incubated at 37° for 4 h in the presence of 1 μg/ml AMD. Cells were pulsed for 30 min with EDC2 + 1 μg/ml AMD with 100 μCi [^{35}S] -methionine.

SECTION I. THE FATE OF INPUT VIRAL PROTEINS

1. Introduction

Kato and Eggers (1969) first reported that adsorption, penetration and uncoating of the virus could all occur at 4°. They measured uncoating by the development of photo-resistant infective centres from FPV tagged with neutral red. Stephenson et al. (in press) extended this work to show that up to 50% of the adsorbed virus became resistant to a pH 3 acid wash of the cells or treatment with neutralizing antibody after 15 min infection at 4°. Furthermore, Stephenson and Dimmock (1975) and Hudson et al. (in press) showed that various components of the input virus reached the nucleus at 4°. It had been observed that the infecting RNA went to the nucleus at 20° and 37° infections (Hoyle and Frisch-Niggemeyer, 1955; Ghendon et al., 1970), but Stephenson and Dimmock also found that 85% of the input [^{32}P]-labelled RNA appeared in the nuclei after 3 h at 4°, and the majority of this was located there within 1 h. On warming to 37°, 75-80% of this nuclear virion RNA returned to the cytoplasm within 30 min, but the rest of the RNA remained associated with the nucleus.

Hudson et al. (in press) confirmed this finding using a different nuclear fractionation technique, but with rather lower nuclear values. This variation might be partly explained by the difference in the efficiency of virus penetration of CEF cells (Stephenson et al., in press). Labelled virion protein also associated with the nucleus at 4° and moved out slowly on raising the incubation temperature to 37°. Experiments with virus labelled with [^{3}H]-choline showed that virion phospholipid remained in the cytoplasmic fraction, a feature confirmed by Metrizamide gradient analysis of the nuclear fraction which showed no [^{32}P] label at the density of phospholipid (1.125 g/ml). Metrizamide analysis of sub-nuclear fractions showed a shift of the [^{32}P] label from the RNP density of 1.255 g/ml to a lower density (1.20 g/ml) after incubation at 37° in all the fractions. It was most marked in the 'RNP enriched' sample, a fraction obtained by extracting nuclei with multiple isotonic salt washes. 1.205 g/ml is also the density at which chromatin bands in this system, thus raising the possibility that a small amount of the RNP becomes associated with cell DNA.

Table 1/1

Input neuraminidase activity in nucleus and cytoplasm

Time after infection (incubation temp. 37°) hpi	NA activity $OD_{550}/h/6 \times 10^8$ hce		Proportion of total recovered activity in nucleus. %
	cytoplasm	nucleus	
0	17.4	0.7	3.9
0.5	15.1	0.5	3.1
1.0	12.2	0.3	2.4
1.5	10.7	0.2	2.0
2.0	7.5	<0.2	
*2.5	65.2	3.5	5.1

* This represents the initial rise of newly synthesized NA and is included for comparison (see also Table 2/2).

In the following experiments the location and movement of the input virion proteins on infection of CEF cells was followed by measuring the activity of the neuraminidase and by determining the fate of prelabelled proteins by analyzing the subcellular fractions by PAGE. Cells were fractionated into nuclear and cytoplasmic extracts by the nuclear monolayer technique. Disrupted nuclei were further analyzed by Metrizamide gradient centrifugation and analysis of gradient fractions by PAGE. The aim of these experiments was to determine the distribution of the input viral proteins following attachment and penetration at 4^{o} and then to compare this with the distribution after incubation at 37^{o} when early viral synthesis had been initiated (Stephenson and Dimmock, 1975). It was intended that this study would throw some light on the organization of the initial events in influenza virus multiplication and the role played by the host cell nucleus.

2. Results

The first stages of virus-cell interaction can be defined as attachment to and penetration of the host cell. The virus then transcribes its RNA which is accomplished with its complement of virion proteins plus host systems. The location and movements of input virus proteins were determined in the following way.

Virus was grown in the presence of [^{35}S]-methionine, partially purified and used to infect cells at 4^{o}. Attachment and penetration occur at 4^{o}, but transcription does not (Stephenson and Dimmock, 1975). The cells were then fractionated and the cytoplasmic and nuclear fractions obtained were analyzed by PAGE. Neuraminidase was located and assayed by its reaction with fetuin (Webster and Laver, 1967).

(2A) Cell Associated Input Viral Neuraminidase Activity

The activity of input viral NA in nuclear and cytoplasmic fractions prepared by the nuclear monolayer technique was determined (Table 1/1). The activity was predominantly (96-98%) cytoplasmic at all times. The first measurement made after infection showed 3.9% of

Table 1/2 Cell associated input viral proteins after 4° infection and following a further 1 h incubation at 37°

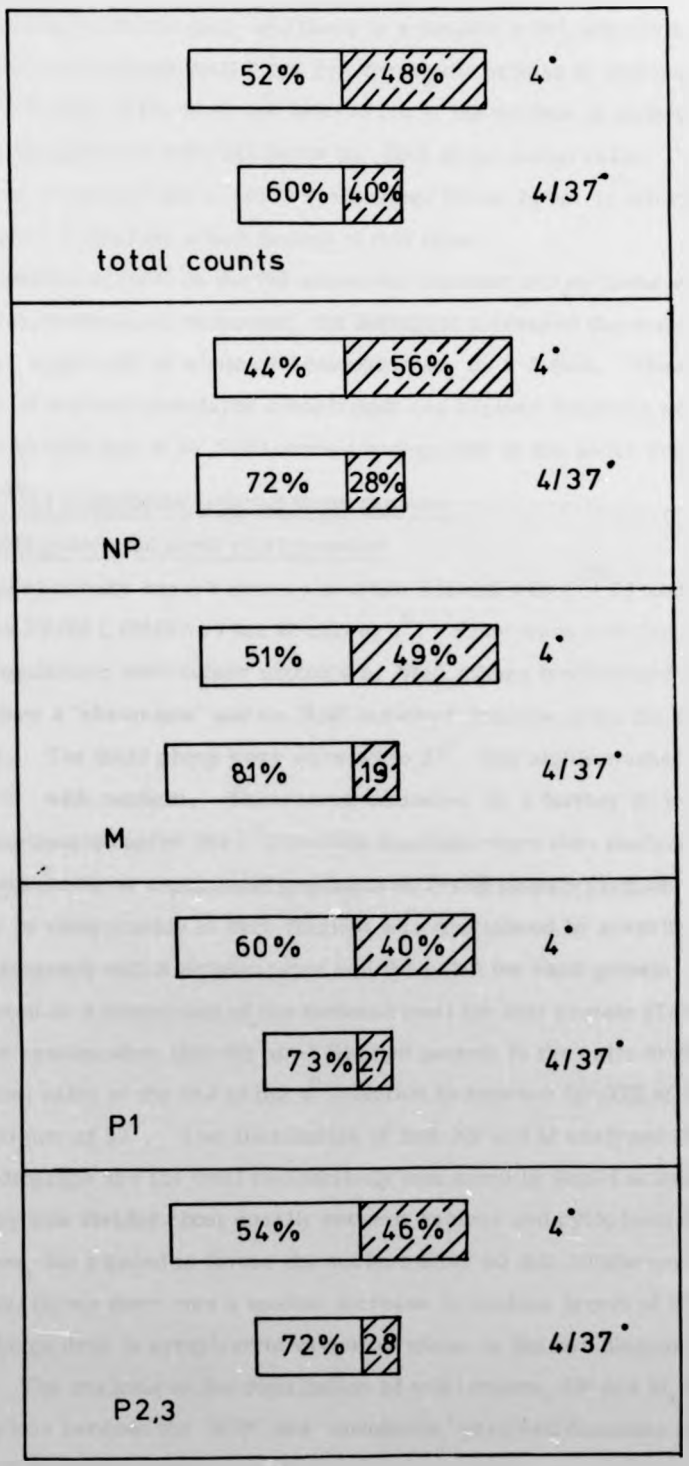
(a) Total labelled input viral protein recovered

	Total [^{35}S] cpm per 10^6 hce	Autoradiograph scan density OD/24 h exposure/10^6 hce			
		P_1	$P_{2,3}$	NP	M
Following 4° infection	14055	0.017	0.032	0.50	0.58
Following 4° infection and incubation for 1 h at 37°	8148	0.008	0.016	0.35	0.45
% viral protein remaining after incubation period	58	47	50	70	78

(b) Distribution between cytoplasm (shaded) and nucleus (clear). Length of bar represents actual remaining radioactive protein whilst % values measure the proportion of that remaining which is in each compartment.

This table shows that:
(1) there is considerable degradation or loss of all input viral proteins after 60 min at 37° (compare length of bars).
(2) The proportion of these proteins in the nucleus increases more substantially than the actual amounts (NP,M compare modest increase in length of bar with large increase in %; P1, P2,3 compare reduction in bar length with increase in %).
(3) This illustrates that at least two processes are occurring concurrently: NP and M are accumulating in the nucleus; a considerable amount of NP and M is being lost from the cytoplasmic fraction.

(b)


52%
48%
4°
60%
40%
4/37°
total counts
44%
56%
4°
72%
28%
4/37°
NP
51%
49%
4°
81%
19
4/37°
M
60%
40%
4°
73%
27
4/37°
P1
54%
46%
4°
72%
28
4/37°
P2,3

total activity in the nucleus, and there is a decline in NA activity in both nucleus and cytoplasm until 2 hpi (mirroring the eclipse in cell-associated virus, see Fig. 2/10) when the level of NA in the nucleus is undetectable and the cytoplasmic level has fallen by 50% of its initial value. The detection of activity due to newly synthesized NA at $2\frac{1}{2}$ hpi is inferred by the 9x rise in total NA which occurs at this time.

The effect of NP40 on the NA assay was checked and as found with NADPH-cytochrome C reductase, the detergent increased the enzyme activity, especially of whole cell homogenates, by 2-3 fold. Thus the NA activity of nuclear monolayer cytoplasmic and nuclear fractions were compared with that of an NP40 treated homogenate in the above study.

(2B) [^{35}S]-methionine Labelled Virus Proteins

(a) Cell associated input viral proteins

In preliminary experiments cells were infected with [^{35}S]-methionine labelled FP/BEL (MOI= 1) for 90 min at 4^{o}. Some were then fractionated into cytoplasmic and nuclear extracts by NML, others fractionated further to produce a 'chromatin' and an 'RNP enriched' fraction from the nuclear extract. The third group were warmed to 37^{o}, thoroughly washed and overlaid with medium. These were incubated for a further 60 min and then fractionated as for the 4^{o} group. The fractions were then analyzed by electrophoresis on exponential gradients of 7-17% polyacrylamide. The content of viral protein in each fraction was quantitated by scanning the autoradiograph with a densitometer and the value for each protein calculated as a proportion of the summed total for that protein (Table 1/2a).

The results show that the total labelled protein in the cells dropped from its initial value at the end of the 4^{o} infection to between 50-80% of this level after 60 min at 37^{o}. The distribution of both NP and M analyzed from the autoradiograph and the total radioactivity measured by liquid scintillation counting was divided about equally between nucleus and cytoplasm after infection, but altered to favour the nucleus after 60 min incubation at 37^{o}. In actual terms there was a modest increase in nuclear levels of NP and M, and a large drop in cytoplasmic values as shown in the bar diagram (Table 1/2b). The analysis of the distribution of total counts, NP and M, within the nucleus between the 'RNP' and 'chromatin' enriched fractions showed that this remained stable at 3:1.

Table 1/3 Distribution of [^{35}S] -methionine labelled input virus proteins after infection at 4° and incubation at 37°

(b) Distribution of radioactivity

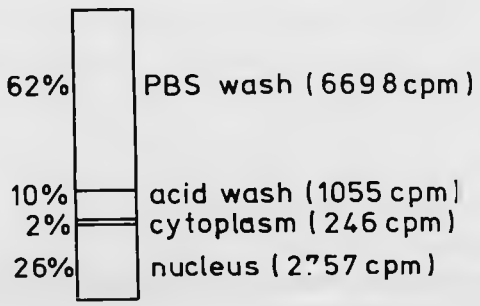
Incubation at 37° (min)	Total cpm	Total radioactivity: % of total cpm recovered				Intracellular radioactivity		
		Wash	Acid wash	Medium	Intracellular	Total cpm	% of cytoplasm	Total nucleus
0	10756	62	10	-	28	3003	8	92
60	10149	62	12	1	24	2472	22	78
180	13496	72	10	2	17	2247	31	69

Table 1/3(a)

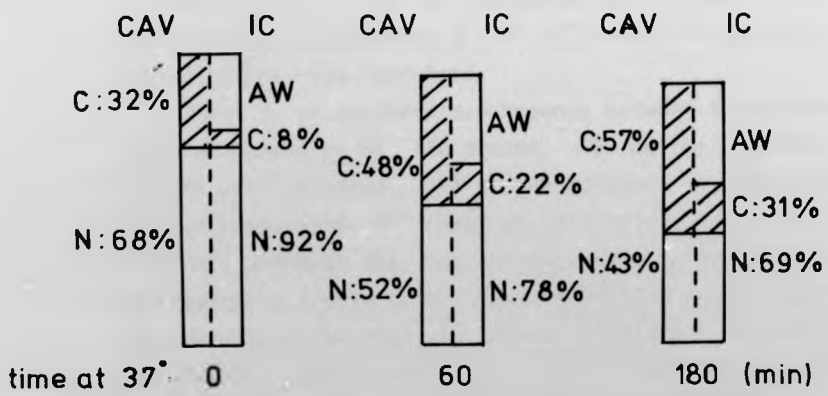
This shows that (1) most (62%) of the virus did not attach to the cells and appeared in the PBS wash; (2) of the cell-associated virus a substantial portion (10% of total) was released by acid wash treatment.

Table 1/3(c)

Attempts to reconcile the observations on cell associated virus (this study; Hudson et al., in press) with those obtained with acid washed cells. It is postulated that as the incubation time increases, the 'cytoplasmic fraction' of CAV approaches that of IC - the other virus being liberated into the medium gradually instead of abruptly by the action of the acid at the end of this infection period.



(a) Proportions of total radioactivity in various fractions following 90 min incubation at 4°



(c) Proportions of putative cell associated counts compared with those of intracellular counts (after acid washing).

CAV - cell associated virus IC - intracellular virus
N - nucleus C - cytoplasm AW - acid wash

(b) The effect of acid wash treatment on the apparent distribution of viral proteins between nucleus and cytoplasm

Infectious influenza virus which adheres to the outer surface of cells during infection can be inactivated by treatment with PBS at pH 3 (Stephenson et al., in press). Virus which has penetrated the cells is not inactivated and will form plaques. However, the fate of the components of the virus was not known. This was investigated by fractionating the cells in the following experiments. The acid wash solutions were pooled, the protein from all samples precipitated with ethanol, and the resulting samples analyzed by liquid scintillation counting and PAGE. Table 1/3 illustrates the results.

The distribution of the total radioactivity was divided between the fractions as illustrated (Table 1/3a). 62% of the input viral proteins do not attach to the cell monolayer and were present in the PBS wash added after 30 min at 4° (see Methods, part 4). The remainder of the radioactivity was divided between the monolayer (28%) and the acid wash (10%). It was found that with the removal of extracellular virions by acid washing, 92% of intracellular virus proteins were in the nucleus after a 90 min incubation at 4° (Table 1/3b). This dropped to 69% after 3 h at 37° whilst the proportion in the cytoplasm rose four-fold.

There is an apparent discrepancy between these results and those of Hudson et al. (in press). Using their method, similar results were obtained. 32% of the radioactivity was found in the cytoplasm after 4° infection, and this rose to 57% after 3 h at 37°, although the total of recovered radioactivity fell substantially (Table 1/3c). Hudson et al. did not acid wash cell monolayers so that the higher cytoplasmic radioactivity was probably due to virus on the outside of the cell which subsequently appeared in the cytoplasmic fraction.

The results of the cell-associated virus experiment reported in this study (Table 1/2) and the drop in total radioactivity observed by Hudson et al. can be reconciled as follows. The apparent cytoplasmic level of input viral proteins was reduced at later incubation times in the CAV experiments by two means. One was the degradation of viral proteins within the cell; the second was the gradual elution into the medium of virus which is adsorbed to the cell surface. This is virus which is removed

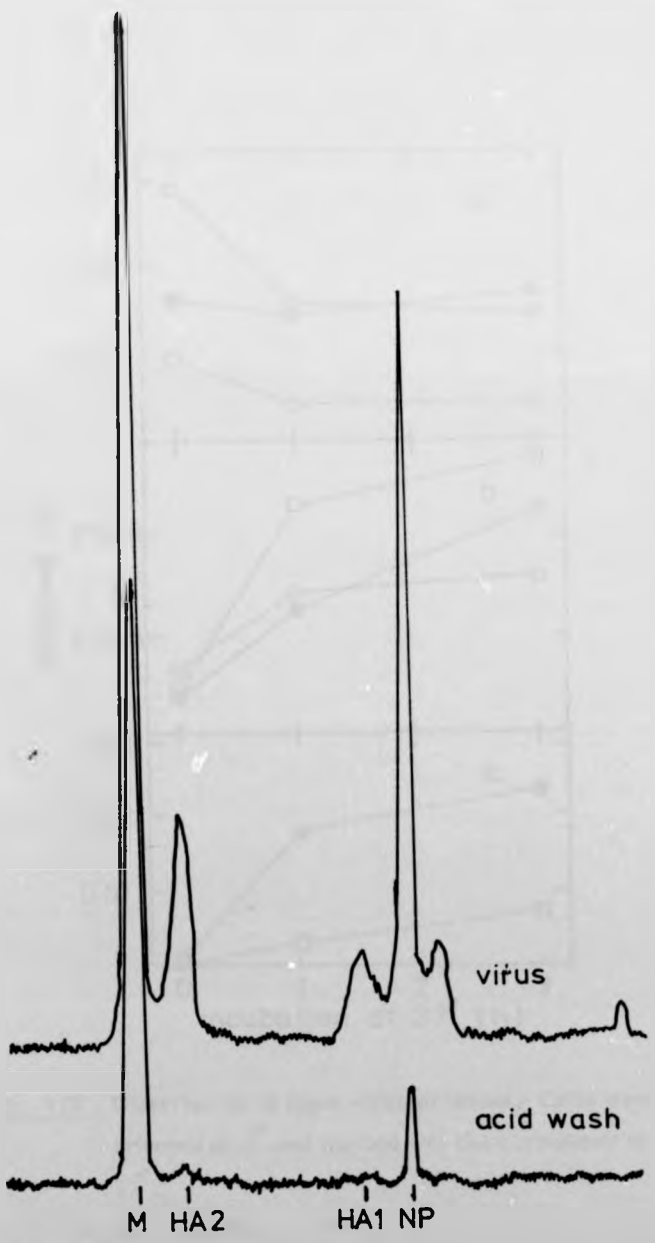


Fig. 1/1 Scanned profile of PAGE of labelled virus compared with acid wash fraction from cells infected with labelled virus at 4^{o}.

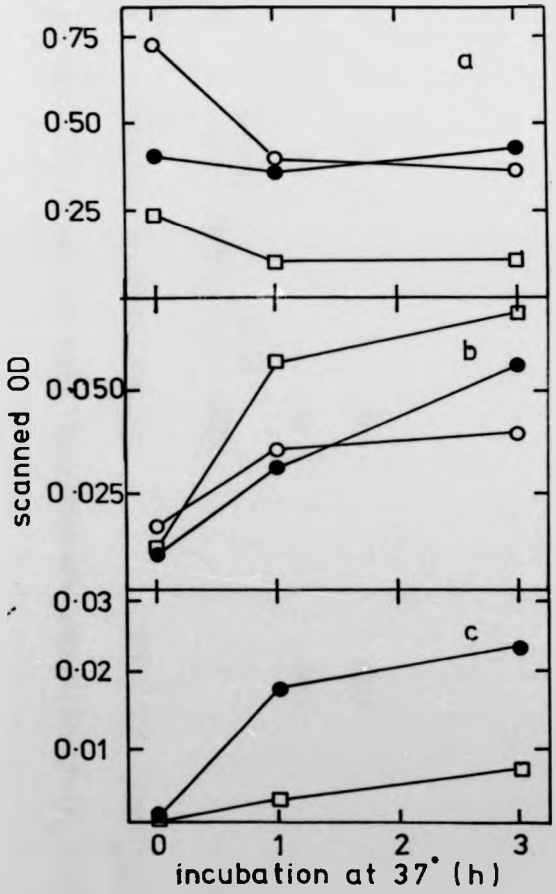


Fig. 1/2 Distribution of input virus proteins. Cells were infected at 4^{o} and washed and then incubated at 37^{o}.

(a) Nucleus ○NP
(b) Cytoplasm ● M
(c) Medium □HA2

Table 1/4

Distribution of [^{3}H]-uridine labelled input virus RNA. Cells were infected for 90 min at 4^{o} and incubated at 37^{o}.

Incubation at 37^{o} (min)	Total cpm	% total radioactivity recovered				% distribution of intracellular radioactivity from time 0		
		Wash	Acid wash	Medium	Intra-cellular	Medium	Cytoplasm	Nucleus
0	1492	61	7	-	32	0	13	87
60	1424	66	6	4	24	14	28	57

immediately by the acid washing technique and thus appears in the acid wash fraction.

Analysis of the acid wash fraction showed it to contain predominantly M and NP. Neither the P proteins nor the glycoproteins were present in more than trace amounts. Fig. 1/1 compares the PAGE profiles of acid wash fractions and whole virus. The ratio of M to NP in the acid wash fraction is over four-fold higher than in the virus.

(c) Distribution and movement of input virus proteins

All the detectable proteins were present predominantly in the nucleus including the glycoprotein HA2 (Fig. 1/2). The levels of NP and HA2 fell to about 50% of their initial value during the first hour at 37° but thereafter remained stable. The level of matrix protein in the nucleus did not alter substantially during the 3 h incubation. As expected from the total radioactivity in the fractions, the levels of all the viral proteins resolved in the cytoplasm were much lower than in the nucleus. Whereas M and NP were predominant in intensity in the nuclear fraction, HA 2 became the most heavily labelled species in the cytoplasm. All the cytoplasmic viral proteins accumulated during the first hour of incubation HA 2 increasing five-fold in intensity whilst the levels of M and NP increased 2-3 times. Over the next two hours, HA2 and NP increased slightly whilst M continued to rise at the same rate as in the first hour. In the medium only M and HA2 were detectable and then only after 1 h incubation. The levels of both proteins rose gradually between 1 and 3 h of 37°.

Whilst the drop in the level of HA2 (53%) in the nucleus is mainly accounted for by its accumulation in the cytoplasm (32%) and tissue culture fluids (4%) and the levels of M remain fairly consistent, the loss of 50% of the initial nuclear NP is unaccounted for. Very little is found in the cytoplasm (about 5%) whilst this protein is not detectable in the medium.

(d) Distribution and movement of RNA of input virus

To correlate the movement of virus RNA with that of viral protein, the fate of input virions labelled with [^{3}H]-uridine was determined (Table 1/4). The uptake was similar to that of [^{35}S]-methionine labelled virus, and the proportion appearing in the acid wash was similar (6-7%). 87% of the intracellular virion RNA

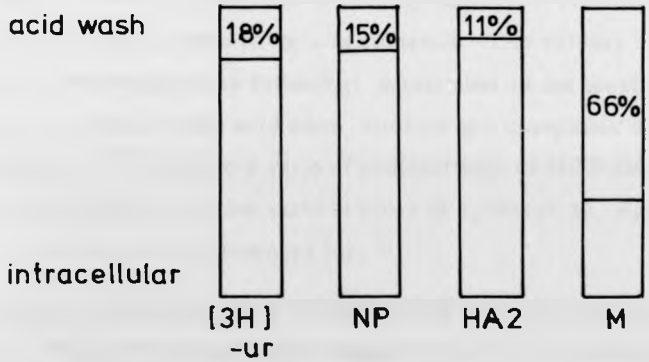


Table 1/5 Distribution of viral components between cells and acid wash.
[^{3}H]-ur Uridine labelled vRNA

appeared in the nucleus after infection at 4°, and the majority of this RNA (66%) remained there after 1 h at 37°. However, 30% of the original RNA left the nucleus and the levels in the cytoplasm and tissue culture fluids rose by equal amounts.

(e) Summary: composition of acid wash

The data on the release of viral components by the acid wash treatment is summarized in Table 1/5. Whereas the proportion of vRNA, NP and HA2 released is roughly equivalent (11-18%), 66% of the cell associated matrix protein is released. The validity of the protein data is enhanced by the following: summation of the levels of each viral protein present in the acid wash, nucleus and cytoplasm after the 90 min infection at 4° leads to a ratio of radioactivity of M:NP:HA2 of 1.46:1:0.32 which compares with the ratio in virus of 1.38:1:0.30, suggesting that all virus protein is accounted for.

Summary: Fate of Viral Components lost from the Nucleus

(a) 34% of the nuclear vRNA migrates out of this organelle, either to the cytoplasm whilst the cytoplasmic RNA is expelled into the medium or roughly equally into cytoplasm and medium.

(b) 50% of the nuclear NP is lost following the incubation period. It does not appear in significiant amounts in either cytoplasm or medium, suggesting that it is degraded within the first hour.

(c) Half of the nuclear HA2 is lost from the nucleus, of which about 70% is found in the cytoplasm, 8% in the tissue culture fluids, the rest presumably being degraded or within the range of variability of measurement.

(d) The level of M remains roughly constant in the nucleus, whilst the level in cytoplasm and medium rises to about 20% of this value. This lack of correlation is not unexpected as it would be difficult to detect the loss of a small amount of M from the high level in the nucleus.

(f) State of input viral proteins within the nuclear fraction: Metrizamide gradient analysis

Two cultures of cells were infected at 4° for 90 min with [^{35}S]-methionine labelled virus, and then acid washed as before, and one was subsequently overlaid with medium and incubated at 37° for a further 60 min.

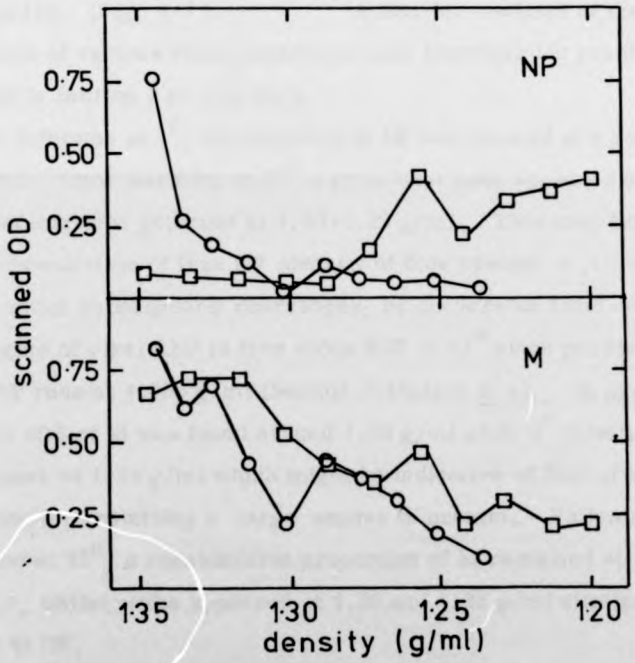


Fig. 1/3 Input viral proteins in infected cell nuclei analyzed on Metrizamide gradients.

- o following 90 min infection at 4°
- □ following infection at 4° and 60 min incubation at 37°

Both cultures were fractionated and the nuclear monolayers scraped and sheared by passage through a fine gauge needle. These samples were then layered over 37% (w/v) Metrizamide and centrifuged to produce a gradient from 1.2 to 1.35 g/ml. The protein from each of the fractions from these gradients was precipitated with ethanol and analyzed by electrophoresis. The peak heights from scans of the resulting autoradiographs were plotted against Metrizamide density for NP and M (Fig. 1/3). A detailed analysis of the distribution of various viral entities on such Metrizamide gradients is presented in Section 3 of this work.

After infection at 4^{o}, the majority of NP was located at a density of 1.35 g/ml. Upon warming to 37^{o} a prominent peak appeared at 1.26 g/ml and a concentration gathered at 1.23-1.20 g/ml. This may reflect either a high concentration of free NP (density of free protein > 1.29 g/ml), some of which subsequently rearranges, or conversion from some form of aggregate of viral RNP to free virus RNP at 37^{o} since purified disrupted virus RNP runs at 1.255 g/ml (Section 3; Hudson et al., in press).

About 60% of M was found around 1.33 g/ml after 4^{o} infection and a further peak at 1.29 g/ml which might be indicative of free protein or some complex containing a large excess of protein. Following incubation at 37^{o}, a considerable proportion of M remained at 1.33 g/ml unlike NP, whilst peaks appeared at 1.26 and 1.23 g/ml similar in density to those of NP.

None of the other viral proteins had sufficient radioactivity to be reproducibly detected. Occasionally HA2 could be seen at 1.26 and 1.23 g/ml. The cytoplasmic samples contained too little radioactivity to be analyzed.

3. Discussion

It appears that 95-98% of the NP and HA2 proteins of input virus which has penetrated the cell at 4° is present in the nucleus. Over 50% of the input matrix protein of virus which has penetrated is exposed on the outer surface of the plasma membrane where it can be liberated by extracellular agents. The remainder of this protein is found in the nucleus. In confirmation of the results of Stephenson and Dimmock (1975) and Hudson et al. (in press), the majority (72%) of input virion RNA is found in the nucleus under similar conditions in these studies. I have reported differences in the movement and fate of viral proteins in the nucleus on warming the infected culture to 37° and have evidence supporting the observation of the above authors that a proportion (34%) of the nuclear input vRNA subsequently moves out at 37°. Finally I have investigated the state of viral proteins in the nucleus and obtained some indication of a rearrangement of viral RNA and proteins. There is a further suggestion of an association of viral components with host cell chromatin (Stephenson, 1974; Hudson et al., in press).

3A. The Initial Events: a. The Appearance of Input Viral Components in the Nucleus

After 90 min infection at 4°, of the cell associated viral components, 72% of the RNA appeared in the nucleus whilst 68% of the proteins were found there. However, only 5% of the proteins and 10% of the RNA was genuinely in the cytoplasm for the remainder was eluted by acid washing. In close agreement, Stephenson and Dimmock (1975) found 70% of cell associated input vRNA in the nucleus whilst Hudson et al. (in press) reported 75%. Consistent with the ribonucleoprotein structure of influenza virus, my studies show 95% of the input NP inside the cell was located in the nucleus but, more surprisingly, so was 92% of the viral glycoprotein HA2. The protein outside the cell (liberated by acid washing) was almost entirely matrix protein and 66% of the total cell associated M was found here.

In contrast, the measurement of input NA activity showed 95-98 % to be in the cytoplasm. The cells in these experiments were not acid-washed but even allowing for a five-fold overestimate of cytoplasmic values by this omission, the ratio of NA in cytoplasm to that in nucleus would still be 85:15. Immunofluorescence studies (see Section 2.2C) showed specks of NA antigen fluorescence in the cytoplasm at 1 hpi and diffuse cytoplasmic staining with bright specks over the nucleus at 1½ hpi. These are consistent with the distribution of NA activity if the specks are in the cytoplasm overlaying the nucleus. Dimmock (unpublished observations) has also observed input NA fluorescence peripheral to the nucleus. The disparity in the location of the virus glycoproteins is surprising considering that they are both incorporated into the virus membrane. Hudson et al. (in press) found that neither [^{32}P] or [^{3}H] choline-phospholipid was found in the nuclear fraction, but always in the cytoplasmic fraction. Thus, if HA2 in the nuclear fraction represented contamination, it would have to be the free glycoprotein and not portions of viral membrane. Although it is possible that the detergent used to fractionate the cells might solubilize the protein, it is invariably observed that newly synthesized HA2 is not found in association with the nuclear fraction prepared by the same method. Alternatively, if the NA activity in the cytoplasm is due to some artefact of the fractionation procedure, then the finding of the phospholipid there must also be spurious.

11-18% of the total cell associated viral RNA, NP and HA2 were recovered in the acid wash and I suggest that these are derived from virions which have adsorbed to but not penetrated the host cell plasma membrane. Of the 66% of matrix protein eluted in this fraction, only an amount equivalent to the other viral components can be derived from adsorbed virions removed by acid washing (i.e. about 15%). The remainder of the matrix protein (about 50% of the total cell associated) must therefore be derived from input virions which have penetrated into the cell. I have constructed a highly speculative scheme which accounts for all these observations but involves mechanisms which appear to be without precedent.

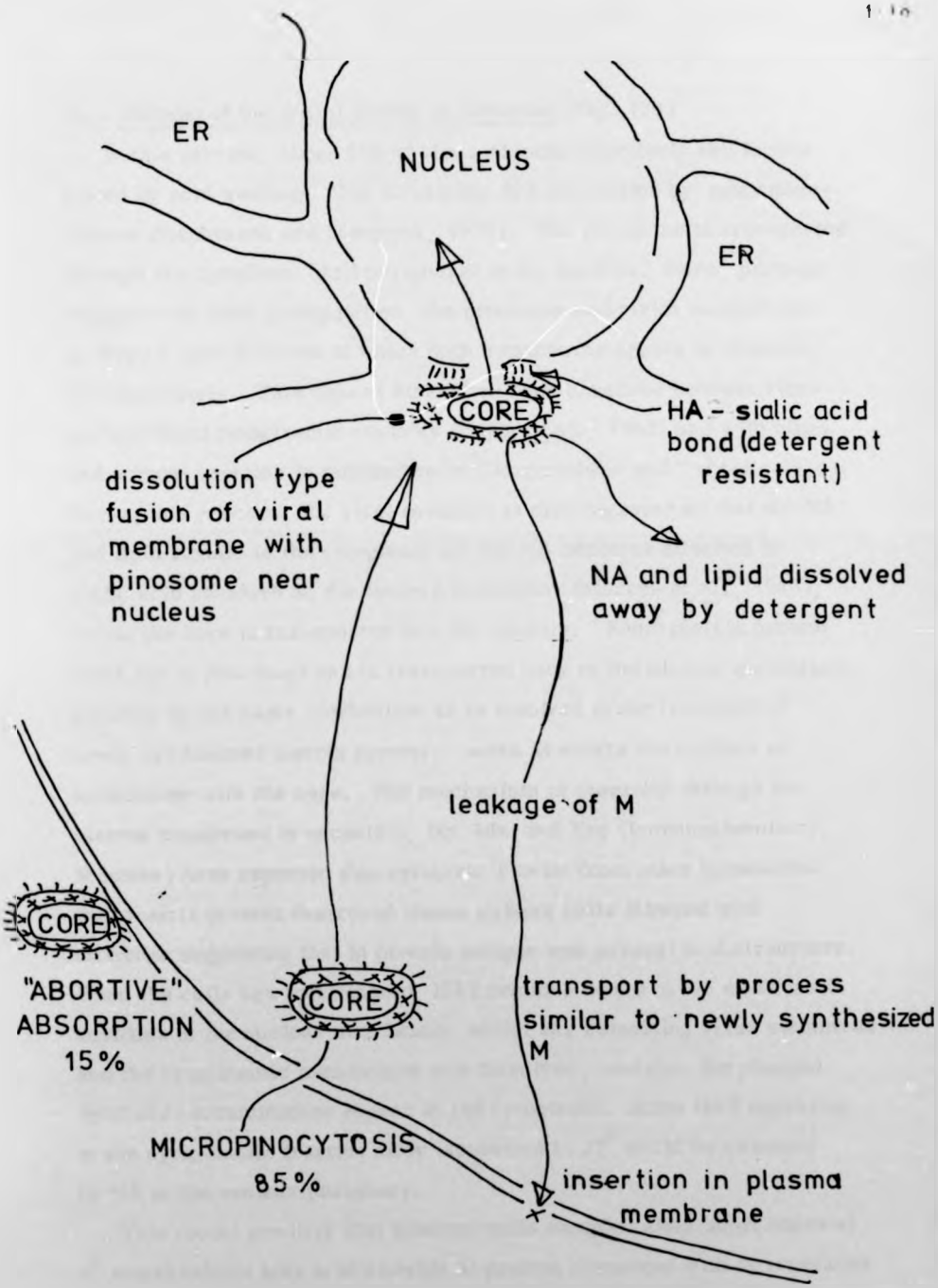


Fig. 1/4 The initial events in influenza virus infection

b. Scheme of the Initial Events in Infection (Fig. 1/4)

In this scheme, about 15% of virus adsorbs abortively and is thus eluted by acid washing. The remaining 85% penetrates by micropinocytosis (Stephenson and Dimmock, 1975). The pinosome is transported through the cytoplasm until peripheral to the nucleus. Here, perhaps triggered by their juxtaposition, the pinosome and virion membranes undergo a type of fusion in which both membranes appear to dissolve simultaneously. This type of fusion has been observed between virus and artificial cytoplasmic vesicles (Hoyle et al., 1962) and with virus and normal vesicles in infected cells (Dourmashkin and Tyrrell, 1974). During this process, the viral envelope is disintegrated so that the NA and lipid remain in the cytoplasm but the HA becomes attached to sialic acid residues on the nuclear membrane (Marcus et al., 1965), whilst the core is transported into the nucleus. Some matrix protein leaks out at this stage and is transported back to the plasma membrane possibly by the same mechanism as is involved in the transport of newly synthesized matrix protein. Some M enters the nucleus in association with the core. The mechanism of insertion through the plasma membrane is uncertain, but Ada and Yap (Immunochemistry, in press) have reported that cytotoxic T cells from mice immunized with matrix protein destroyed tissue culture cells infected with influenza suggesting that M protein antigen was present on their surface. When the cells are fractionated, HA2 remains bound to the sialic acid residues in the nuclear membrane, whilst any remaining virus membrane and the cytoplasmic membranes are dissolved, and thus the phospholipid and neuraminidase appear in the cytoplasm. Some HA2 appearing in the cytoplasmic fraction after incubation at 37° could be released by NA at the nuclear periphery.

This model predicts that infected cells sampled after short times at 4° would exhibit less acid elutable M protein compared with intracellular M than after long periods due to the time to pass through the cytoplasm and become inserted through the membrane. It predicts that the HA2-sialic acid residue bond is resistant to detergent. In infection at 4°

with virus containing non-functional NA, the model predicts that HA2 will not be released into the cytoplasm. The model does not explain how the fusion process can take place at 4^{o} unless the lipids involved are more fluid than those at the plasma membrane where low temperature inhibits fusion. The union of HA2 molecules to sialic acid residues must be remarkably comprehensive to render so little soluble to the detergent. M may be inserted into or through the plasma membrane by the processes which incorporate cellular proteins.

3B. The Movement of Input Viral Components after Warming to 37^{o}

A loss was observed of 34% of the nuclear vRNA and 50% of the NP and HA2 from the nucleus. There was little change in the pool of M. Hudson et al. (in press), using the same NML technique, reported 50% of the vRNA migrated whilst Stephenson and Dimmock (1975) found 75-80% by conventional fractionation. Thus these results agree that a substantial proportion of the input vRNA leaves the nucleus at a stage when the initial transcription is beginning.

70% of the released HA2 in this study reappears in the cytoplasm, 8% is ejected into the medium, and the rest is unaccounted for, presumably degraded. There is no sign of additional NP in either cytoplasm or outside the cell to correspond to the 50% loss from the nucleus which could mean that the NP is degraded in the cytoplasm.

There is some evidence, discussed below, to suggest that a large proportion of NP in the nucleus may be free. Although the preliminary experiments of this study did not show a decrease in NP in the nucleus, the overall level of NP dropped markedly so degradation could be involved. Hudson et al. (in press) found a large decrease in the viral protein in the nucleus which could reflect transport out of the nucleus and degradation in the cytoplasm.

The movement of RNA and NP from the nucleus may be a process specific to virus multiplication, particularly since the level of M protein in the nucleus remains substantially unchanged. As discussed above, I think that the movement of HA2 is a separate process, unconnected with multiplication, resulting from the cleavage of HA2 from the sialic residues on the nuclear membrane.

3C. The State of Input Viral Proteins in the Nucleus

This study showed that 60% of both NP and M were initially at high density (1.32-1.35 g/ml) after infection at 4° and centrifugation on metrizamide, but on warming to 37° that peaks of NP and M appeared at 1.255 g/ml which is around the density of vRNP's (Section 3.2; Hudson et al., in press) and at 1.23-1.21 g/ml at which density chromatin bands in this sytem. Some M remained at high density after the 37° incubation, but all the NP disappeared. I would tentatively suggest two schemes that explain the data.

(1) NP is initially removed from the vRNA and thus bands at the density of free protein (> 1.29 g/ml). Some M remains in association with the viral RNA but much is free also. After 37° incubation, the NP reassociates with the RNA, more M reassociates with RNA and some of these vRNP complexes become associated with cell chromatin at 1.23-1.21 g/ml.

(2) After 4° infection, the viral RNP appears to be associated with extra viral protein (mostly NP) which would increase the density in metrizamide above the 1.255 g/ml. On centrifugation, some of these extra proteins form complexes with metrizamide, such as has been reported for free proteins (Rickwood et al., 1974a), and this accounts for the increase in apparent density of the vRNP complex to > 1.30 g/ml. On warming to 37° these extra proteins are removed, and the vRNP reverts to its normal density of 1.255 g/ml and possibly associates with chromatin. There is an excess of M and so the loss from high densities on stripping of this complex is obscured. In support of this theory is the slight peak of M and NP at 1.29 g/ml after 4° incubation which may represent the vRNP -protein complex without attached metrizamide.

However, the data are insufficient to support either scheme strongly, and the structures observed at 4° infection may not be 'real' intermediates in the virus growth cycle under normal conditions.

4. Conclusion

Our results suggest that influenza viruses are uncoated at some site close to the nuclear membrane. Further experiments are needed to determine exactly where the virus loses its lipid envelope since this is the crucial evidence that virus is not uncoated by fusion with the nuclear membrane. A more detailed knowledge of the fate of the NA and HA is also needed so that the significance of their distribution between nucleus and cytoplasm can be determined. It is hoped to be able to label the glycoproteins with [^{125}I] so that the fate of these proteins after infection will become apparent. However, there may well be problems of iodinating and maintaining infectivity.

The acid wash technique, introduced to remove virus attached to the outside of the cell, and hence to obtain a more realistic value for proteins in the cytoplasm,has proved a useful tool in improving the study of the penetration of virus into cells.

The suggestion that viral RNP associates with the chromatin in the nucleus is of interest in regard to the requirement for a nuclear function to initiate viral multiplication (Barry et al., 1962; Follett et al., 1974; Spooner and Barry, 1977). Clearly further investigation is required into the nature of this association, and what that means in functional terms. In other words, is such an association fortuitous or is it an essential feature of multiplication?

This section has shown many of the difficulties involved in a biochemical study of a complex biological phenomenon and the difficulty of reaching unambiguous answers to the questions posed. However, something has been learned about the distribution of the viral proteins and the way has been pointed for further experimentation.

SECTION 2

Location and Movement of Newly Synthesized Viral Proteins

1. Introduction

In previous studies there has been some disagreement over the newly synthesized viral proteins which are found in the nucleus. Taylor et al. (1969, 1970) found what was thought to be M in the nucleus, Lazarowitz et al. (1970) reinterpreted this as NS1 whilst the work of this group and Krug's studies (Krug and Etkind, 1973; Krug and Soeiro, 1975) were in agreement that matrix protein remained cytoplasmic. Gregoriades extracted M from the nucleus (Gregoriades, 1973), Hay and Skehel (1975) reported it to be accumulated there whilst the behaviour of matrix antigen was shown to be variable (Oxford and Schild, 1975).

There has been a concensus that viral proteins could not be observed to migrate out of the nucleus (Krug, 1972; Krug and Etkind, 1973; Hay and Skehel, 1975) although the behaviour of the virion RNP antigen has been interpreted as demonstrating the migration of NP into the cytoplasm (Breitenfeld and Schafer, 1957; Maeno and Kilbourne, 1970; Kelly and Dimmock, 1974). However, the significance of this migration is completely unknown.

It was in an attempt to resolve some of these differences and to illuminate the role of influenza virus proteins in the nucleus that this study was initiated. It was hoped to resolve the apparent paradox between the movement NP antigen and the lack of movement of labelled NP from the nucleus. The involvement of nuclear viral proteins in the nuclear step and whether they were subsequently incorporated into virions was also investigated. These studies were extended to cover the release of all protein components into the tissue culture fluids. I was particularly interested to see whether proteins not incorporated into virions were released differentially or at random. Previous studies on the release of virions had considered only the time of synthesis of viral proteins leading to their maximal incorporation into virions (Meier-Ewert and Compans, 1974; Krug and Etkind, 1973) and the kinetics of incorporation of proteins into virions (Hay, 1974).

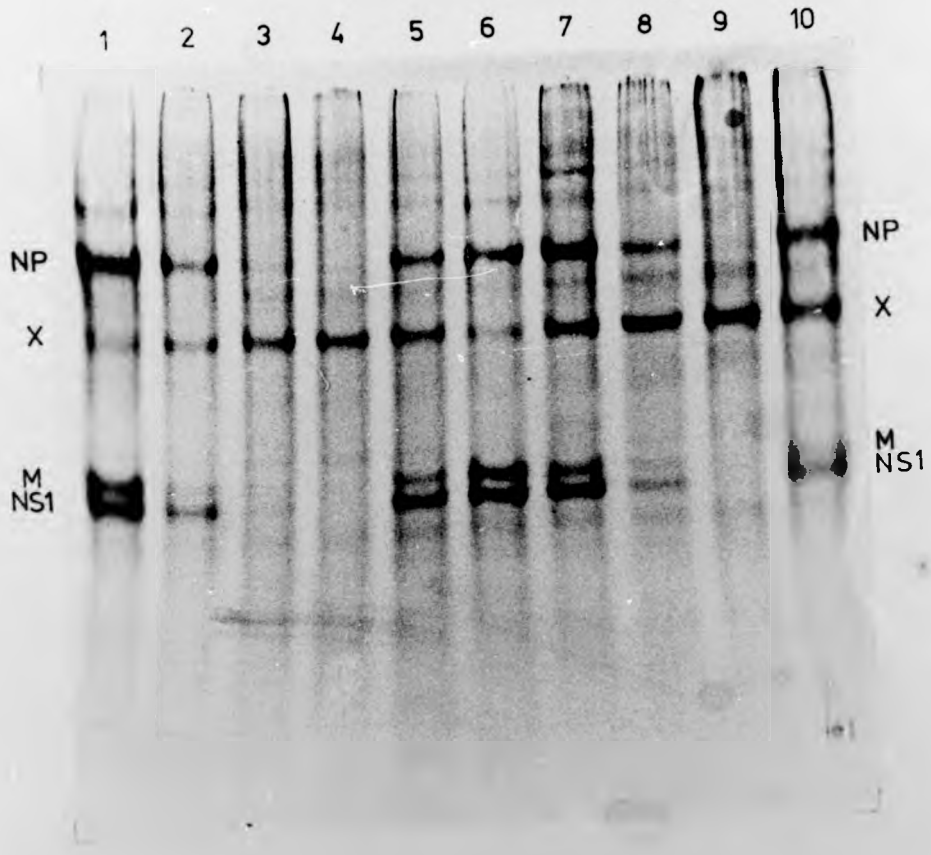
Fig. 2/1 The effect on virus growth at 31° of the temperature and multiplicity of infection.

All cultures were pulsed at 4 hpi with [^{35}S] methionine for 5 min. 1-6 were immediately prepared for PAGE (10% phosphate) whilst 7-10 were chased in medium containing cold methionine for 20 min.

		Infection temp (°C)	Chase (min)	MOI
1	Marker	18	0	100
2		4	0	100
3		4	0	10
4		4	0	1
5		4	0	100
6		18	0	100
7		4	20	100
8		4	20	10
9		4	20	1
10	Marker	4	20	100

2, 3, 4 illustrate the effect on viral protein synthesis, in a 5 min pulse, of decreasing MOI with 4° infection, and 7, 8, 9 similarly with a 20 min chase following the pulse. 5, 6 compare the relative density of protein bands after 4° and 18° infection.

X is the host protein actin



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2. Intracellular: the Synthesis and Distribution of Radiolabelled Viral Proteins

2A. Pulse-chase Experiments: Techniques

(a) Growth of influenza virus at 31°

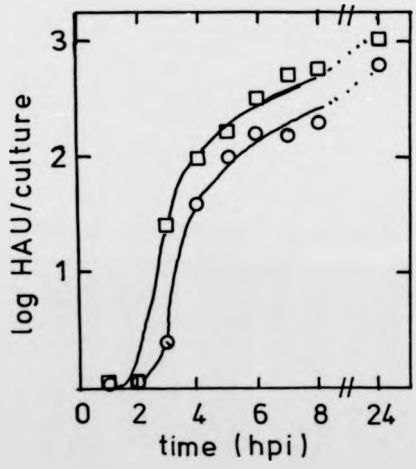
A preliminary investigation into the growth of influenza virus at 31° C was carried out in order to determine the feasibility of a study of the movements of radiolabelled viral proteins between cytoplasm and nucleus. Incubation at lower temperature was used to extend the time course of protein synthesis since Skehel (1972) had shown that early and late proteins could regularly be distinguished at 31° though not at 37°. The correlation of antigen movements with those of newly synthesized virus proteins detected by radiolabelling would thus be facilitated. By infecting with the virus at 4° it was hoped to improve the synchrony of virus infection since the majority of infecting virus in the cells would have completed the attachment, penetration and uncoating stages (Stephenson et al., in press). A multiplicity of infection of 1-5 PFU/cell was used initially to avoid the possible anomalous effects of high multiplicity leading to the production of von Magnus virus. According to Skehel (1972) at moi 2, all viral proteins were detectable by radiolabelling.

Cultures of CEF cells were infected with FPV (moi 2) for 30 min at 4° and incubated for 4½ h at 31°. Neither a 5 min pulse of [^{35}S]-methionine nor a pulse followed by a 25 min chase produced any detectable viral protein on polyacrylamide gels.

The autoradiograph (Fig. 2/1) compares both the effect of 4° and room temperature infection and different moi on infected cells, either pulsed with [^{35}S]-methionine at 4 hpi for 5 min or pulsed and then chased for 20 min. The chase strengthens viral protein bands but after 4° infection with moi 1, virtually no virus NP was produced and the intensity of viral bands increased in samples infected at 10 to 100 PFU/cell. Note that the host protein, actin (X), is always labelled.

After room temperature infection, the virus growth cycle was further advanced as judged by the relative intensity of M and NS1 (see below, 2A.(b)). Intracellular HA production was also earlier after room temperature than after 4° infection (see 2A.(b) and Fig. 2/2). Cells

Fig. 2/2 Intracellular HA production at 31°
○ infection at 4°. □ infection at room temperature.



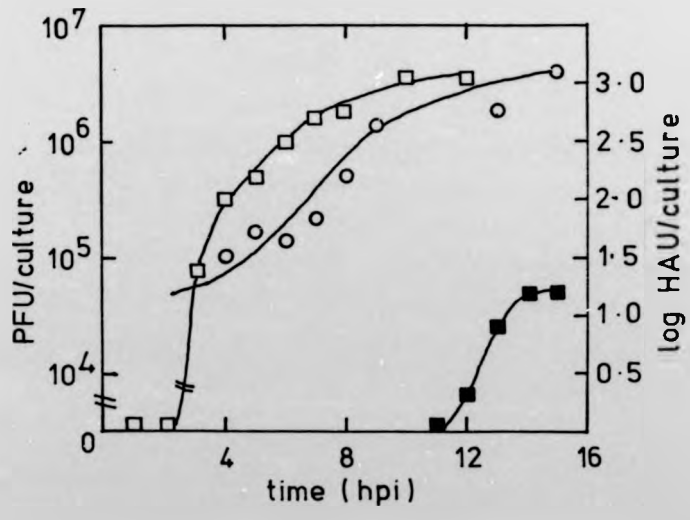


Fig. 2/3 Virus growth parameters in infected cells incubated at 31°.
□ intracellular HA
○ PFU released
■ HA released

Fig. 2/4 The synthesis of viral proteins in infected cells at 31°.

Cultures were pulsed at the times indicated for 5 min with [^{35}S] methionine, and then processed for PAGE on a 10% phosphate gel with 15% "stopping layer" as indicated.

X is the host protein actin.

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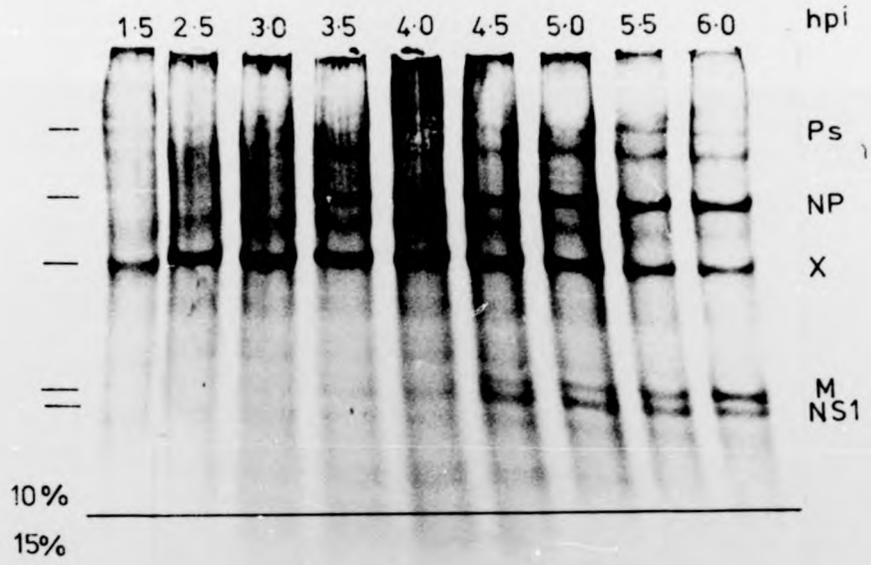


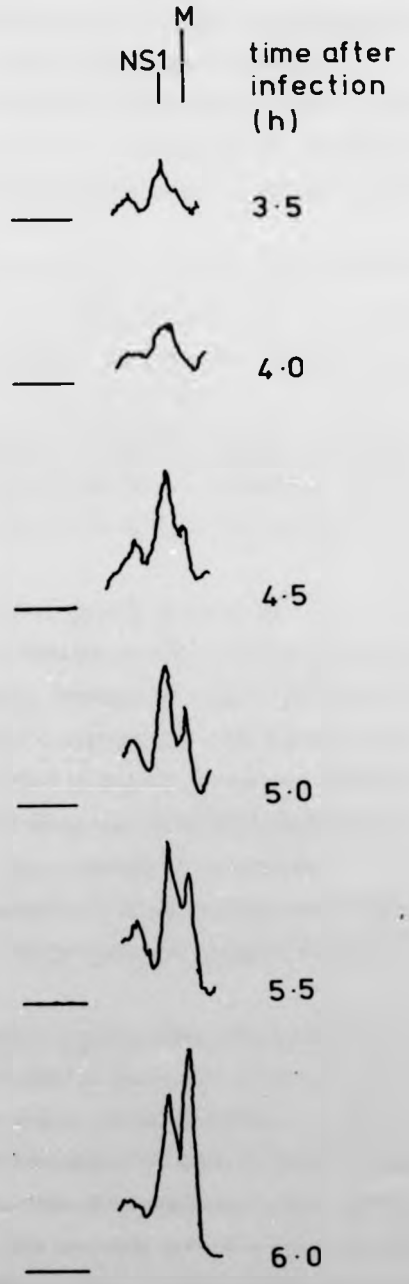


Fig. 2/5 The appearance of M and NS1 in infected cells incubated at 31°. Profiles are scans of an autoradiograph of a 10% phosphate gel. The horizontal line left marks the baseline. Cells were infected for 15 min at room temperature and incubated at 31°. At times indicated, cells were pulsed for 5 min with [^{35}S] methionine and immediately prepared for PAGE.

infected at 4° with moi 10 eventually produced a faint trace of NP when pulsed after 6 h incubation at 31°. This contrasted with considerable NP at 3½ hpi after a room temperature infection.

Intracellular HA produced after infection with moi 100 at both 4° and 20° is shown in Fig. 2/2. Though there was about a 1 h time lag between the HA production of the cultures, the final yields of HA were similar.

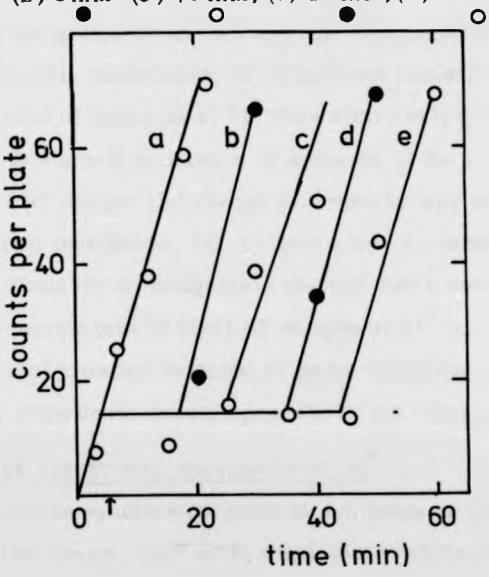
The combination of low moi, 4° infection and 31° incubation did not produce sufficient virus protein for analysis. At higher moi and infection at 4° variable results were obtained which probably depended on the batch of primary CEF cells used which vary in their efficiency of virus attachment and penetration (Stephenson et al., in press). These conditions were therefore abandoned. Instead the maximum synchrony of infection was obtained by infecting cells for only 15 min at room temperature.

(b) Parameters of virus growth cycle at 31°

The production of infectious virus, intracellular and extracellular HA was measured following infection of cells at 20° with moi 15 (Fig. 2/3). Released haemagglutinin activity was only detectable after 12 h whilst intracellular HA reached its maximum activity between 8-10 h. The majority of infectious virus was released between 6 and 9 hpi, about 2 h later than at 37°. The synthesis of viral proteins was first detectable at 3½ hpi with the appearance of NP and a trace of NS1 (Fig. 2/4 and 2/5), M became visible at 4½ hpi and more intense than NS1 by 6 hpi which agrees with Skehel (1972).

Immunofluorescence studies using monospecific anti-NP serum (prepared according to Kelly and Dimmock, 1974) showed that nuclei accumulated NP antigen from 4 to 5 hpi whilst the cytoplasms remained empty. A diffuse annulus of stain peripheral to the nucleus appeared from 7-9 hpi whilst the interior was less intensely stained than the cytoplasm. Some cells showed dark spots over the nucleoli. This was only seen in a minority of cells, however, most exhibiting strong nuclear fluorescence until 11-15 hpi when the balance of staining intensity shifted from the nucleus in favour of the

Fig. 2/6 Recovery of protein synthesis following salt-shock. Infected cells at 5 hpi/31°C were pulsed at time = 0. At time = 5, all cultures apart from controls (a) were subjected to 150 mM NaCl. o Cultures were released from salt after various times: (b) 5 min · (c) 15 min; (d) 25 min; (e) 35 min. ↑ marks salt-shock.



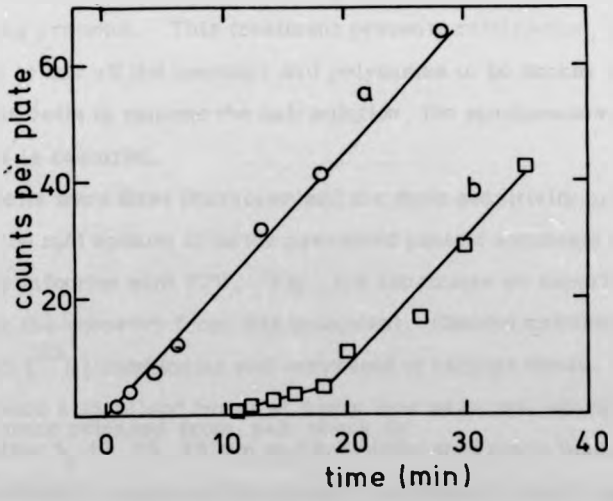


Fig. 2/7 Fine detail of recovery of protein synthesis following salt-shock.
(a) o control. (b) □ cells salt-shocked at time = -20 and released at time = 10.

cytoplasm. The state was reached when whole cells fluoresced so intensely that the nuclei could no longer be distinguished.

The asymmetric distribution of NP between nucleus and cytoplasm during the course of infection at 31° thus alters only gradually in most of the cells. In a small population of about 5% of the culture the distribution of NP antigen did change in a similar way to that observed at 37° incubation (see below, 2C.) albeit about 2 h later.

These preliminary investigations showed that it was not feasible to correlate the movements of viral NP antigen at 31° with those of newly synthesized viral proteins detected by pulse-labelling since the movement of the former were continuously spread over the virus growth cycle.

(c) Salt shock synchrony: application at 31°

In an attempt to synchronize protein synthesis to allow the use of very short pulse times, CEF cells were characterized for their sensitivity to salt shock. This is the phenomenon (Saborio et al., 1974) in which cells exposed to medium containing sodium chloride stop synthesizing proteins. This treatment prevents reinitiation, causing ribosomes to run off the message and polysomes to be broken down. By washing the cells to remove the salt solution, the synchronous synthesis of proteins is restored.

CEF cells were first characterized for their sensitivity to salt shock at 31°. 150 mM sodium chloride prevented protein synthesis in cells 4-5 h after infection with FPV. Fig. 2/6 illustrates an experiment to investigate the recovery from this treatment. Control cultures were pulsed with [^{35}S]-methionine and harvested at various times. All other cultures were also pulsed but after 5 min they were salt-shocked. Cultures were released from salt shock by washing after 5, 15, 25, 35 min and harvested at various times.

The cells still recovered the ability to synthesize protein at or near to control rates after 35 min of exposure to salt. However, there is a noticeable delay between removing the salt and the renewal of protein synthesis which was investigated further (Fig. 2/7). Two sets of cultures were taken after 4 to 5 h of infection. One set was treated with

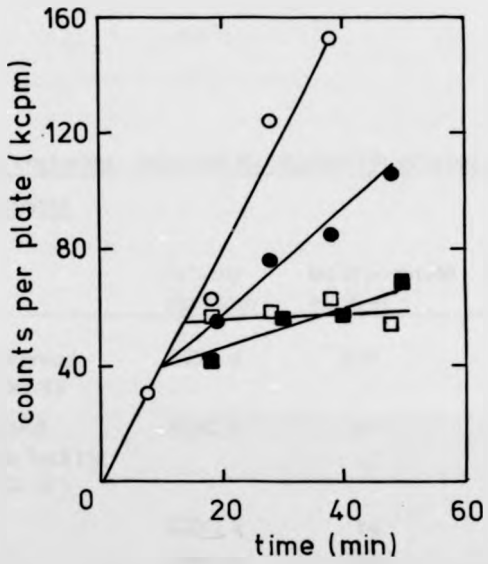


Fig. 2.8 Concentration of NaCl required to inhibit protein synthesis in infected cells at 37°. Infected cells were pulsed at $3\frac{1}{2}$ hpi (time = 0) and salt-shocked at time = 10 to a final concentration of NaCl:

○ Control; no salt. ● 100 mM ■ 150 mM
□ 200/250 mM

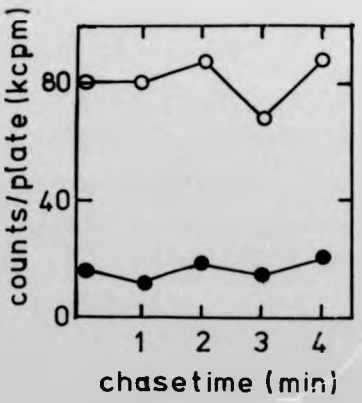


Fig. 2/9 Validity of chase. Infected cells were salt-shocked after 5 hpi at 31° and pulsed (after rescue) for 2 min. Cells were fractionated by NML after the chase times indicated, and processed by hot TCA method.

○ cytoplasm ● nucleus

Table 2/1

Analysis of pulsing regimens: relative incorporation of label into TCA precipitable counts

No.	Starvation medium	Pulsing medium	Incorporation as % of 1	Incorporation as % of 4
1	Earles + 4% dialysed calf serum (EDC 4)	EDC 4	100	
2	Glasgow modified growth medium lacking methionine (GM-M)	EDC 4	56	
3	–	EDC 4	14	
4	EDC 4	GM-M	20	100
5	GM-M	GM-M	17	85
6	–	GM-M	7	36

150 mM sodium chloride for 30 min and then washed. Both cultures were pulsed with radioactive methionine. TCA-insoluble counts from cultures, harvested at various times, were recorded.

Although the salt-treated cells attain the rate of protein synthesis of the control cultures, there is a lag of 8 min before this rate is achieved. In other experiments this was found to vary between 8 and 11 min. The lag period between washing out the salt and the achievement of normal rates of protein synthesis made the procedure unsuitable for its original purpose as protein synthesis would no longer be synchronous if the start of the pulse had to be delayed for this lag period.

(d) Salt shock synchrony: its application at 37^{o}

At 37^{o} it was found that in order to inhibit protein synthesis, the concentration of sodium chloride had to be increased. In one experiment sets of cultures were treated with a series of salt concentrations which showed that 200 mM sodium choride was required to inhibit protein synthesis to the same extent as achieved with 150 mM at 31^{o} (Fig. 2/8).

This technique was used to examine the location and movement of influenza viral proteins immediately after synthesis. However, on comparison with the results from longer pulses carried out without the salt shock treatment, it appeared that little extra resolution was gained by this treatment. There also appeared to be some inhibitory effect on glycosylation since bands were observed migrating in the same position as those found by Klenk et al. (1974) after inhibition of glycoprotein synthesis in infected CEF cells with glucosamine and deoxyglucose. It was concluded that, without tangible gains in resolution, salt shock was an unnecessary and potentially disturbing process to impose on the natural virus growth cycle when observing changes over periods of longer than 5 min.

(e) Pulse-chase procedures

In an attempt to maximise the incorporation of radiolabelled methionine into viral proteins, a number of pulsing regimes were investigated. We found that incubation of the cells in pulse medium of Earles with dialysed

Fig. 2/10 Virus proteins synthesized at 1.5 and 2 hpi, in cytoplasm and nucleus. Infected cultures were pulsed with [^{35}S] methionine for 10 min at times indicated and chased for 0, 10 min or to 6 hpi. They were then fractionated by NML into cytoplasm and nucleus and analyzed on 10% phosphate PAGE.
X is the host protein actin.

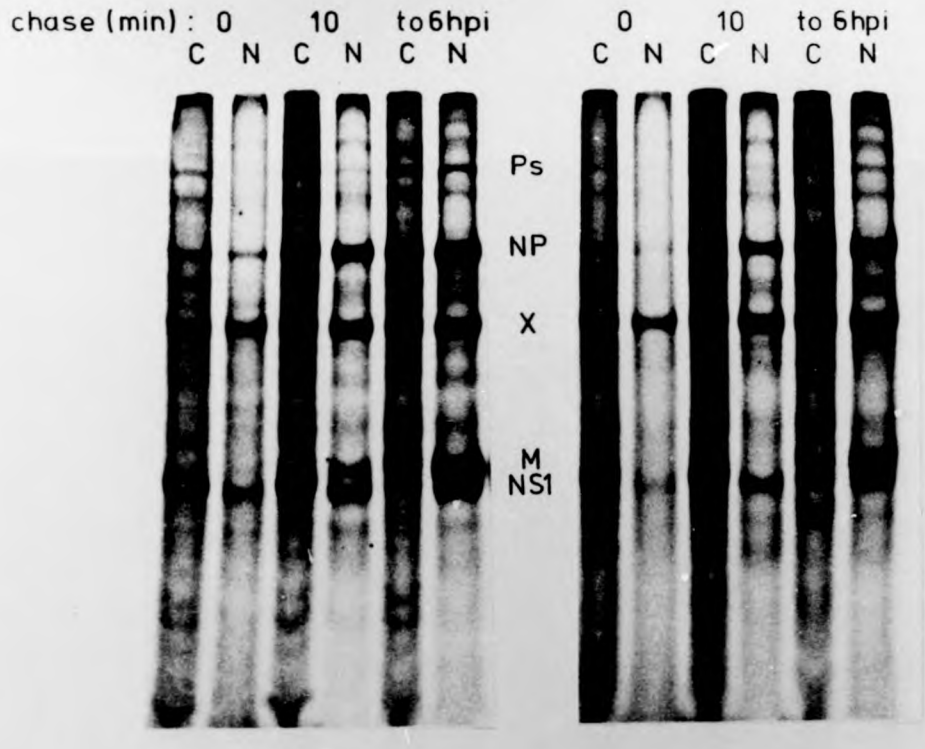
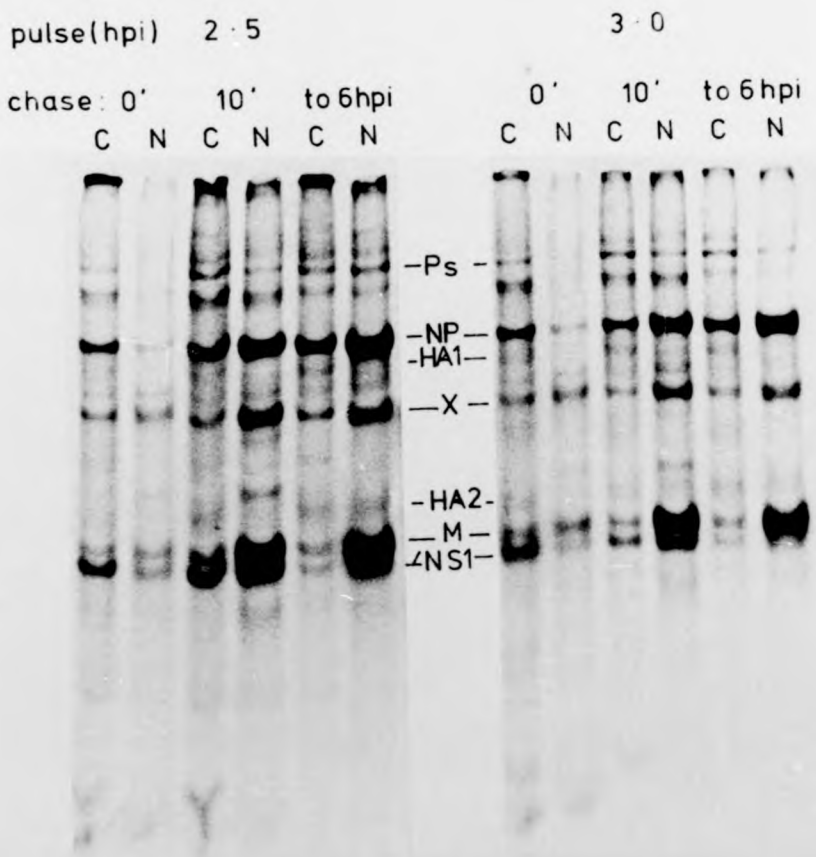

pulse (hpi)
1·5
2·0
chase (min): 0
10
to 6hpi
0
10
to 6hpi
C N C N C N
C N C N C N
Ps
NP
X
M
NS1

Fig. 2/11 Virus proteins synthesized at 2.5 and 3 hpi in cytoplasm and nucleus.
As 2/10.


pulse(hpi) 2·5
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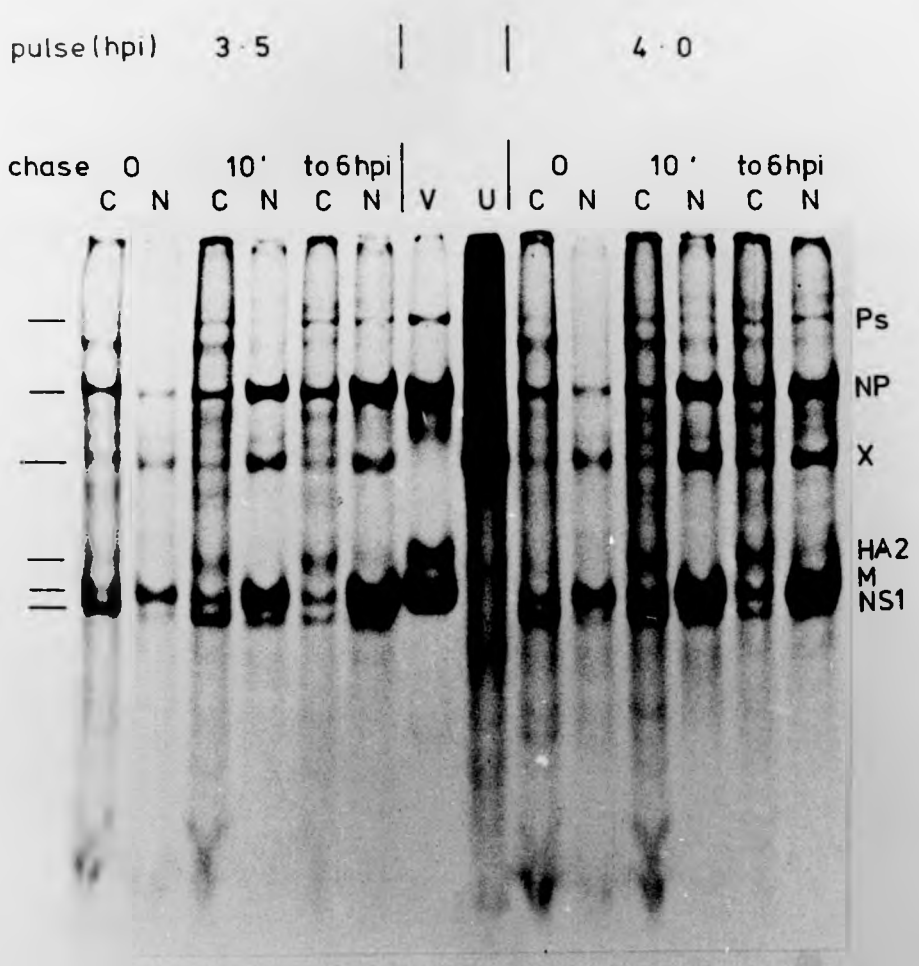
to 6hpi
C N C N C N
0'
10'
to 6hpi
C N C N C N
-Ps -
-NP-
-HA1-
-X-
-HA2-
-M-
-NS1-

Fig. 2/12 Virus proteins, synthesized at 3.5 and 4.0 hpi in cytoplasm and nucleus. As 2/10.


pulse (hpi) 3·5
4·0
chase 0 10' to 6 hpi 0 10' to 6 hpi
C N C N C N V U C N C N C N
Ps
NP
X
HA2
M
NS1

calf serum (EDC4) which lacked radioactive methionine for 10 min before adding the isotope increased incorporation seven-fold (Table 2/1). A similar procedure with Glasgow modified growth medium lacking methionine (GM-M) improved the incorporation 2.5-fold. The reason for this improvement of incorporation with EDC4, lacking all the amino acids, over GM-M, containing a full complement with high specific activity methionine, is unknown. However, incorporation was linear for at least 30 min as shown in the control of Fig. 2/7.

Chase procedures were also investigated. Two washes after labelling with excess Glasgow modified growth medium plus 4% calf serum followed by incubation in that medium resulted in cold TCA precipitable counts rising by 18% in the first 10 min of chase. No further increase in TCA precipitable counts was observed over 1 h of chase. Another illustration of the validity of the chase is given in Fig. 2/9 in which, after salt shock and starvation, a 2 min pulse was followed by a series of chases. The cells were fractionated and treated with hot TCA to hydrolyze labelled aminoacyl-t-RNA's. Precipitable radioactivity was collected on filters. There was no substantial change in cytoplasmic or nuclear counts over the 4 min of chase.

Pulse Chase Experiments: Qualitative Survey of the Location and Subsequent Movement of Viral Proteins Synthesized between $1\frac{1}{2}$ and 4 hpi Since the first large increase (10^3-fold) in intracellular infectivity occurred at 2-$3\frac{1}{2}$ hpi (see Fig. 2/21) time points were taken from $1\frac{1}{2}$ to 4 hpi at 30 min intervals. Sets of three cultures were starved and pulsed, and one plate was fractionated into nucleus and cytoplasm at the end of the pulse, one after a 10 min chase and the final culture at 6 hpi. The cytoplasmic and nuclear extracts were analyzed by PAGE (Fig. 2/10-12).

(a) NP

The appearance and location of NP remained unchanged throughout the period under study except for the 2 hpi time point. Generally more than half of the NP was observed to migrate rapidly to the nucleus. There was no change between its concentration after a 10 min chase and after the

chase to 6 hpi. Its synthesis was well under way at $1\frac{1}{2}$ hpi and remained easily detectable at all time points. At 2 hpi it appeared that the movement of NP into the nucleus was slowed after the 10 min chase, but this observation was not routinely made.

(b) M

This protein was only just visible on the gels after the 10 min chase following labelling at $1\frac{1}{2}$ and 2 hpi. It appeared as a strong band in the nuclei only after the chase to 6 hpi which presumably means that protein synthesis incorporated radiolabelled methionine during this extensive chase. By $2\frac{1}{2}$ h, however, the protein was found in a predominantly nuclear location and by 3 hpi M was observed in the nuclei of cells fractionated without a chase

(c) NS1

In contrast to matrix protein, the non-structural NS1 was observed at $1\frac{1}{2}$ hpi, the earliest time point, and was more intense than NP at this time. NS1 synthesized up to 3 hpi migrated to the nucleus. At $3\frac{1}{2}$ and 4 hpi its synthesis was reduced, and it remained predominantly cytoplasmic in location. The migration of NS1 synthesized from $1\frac{1}{2}$ to $2\frac{1}{2}$ hpi into the nucleus was slower than that of NP and M since after 10 min of chase the labelled protein was still predominantly cytoplasmic, though with a longer chase to 6 hpi the majority moved to the nuclear fraction. At 3 hpi it seemed that the movement of NS1 was faster as the label was predominantly nuclear after a 10 min chase whilst at later times nuclear accumulation did not occur.

(d) P proteins

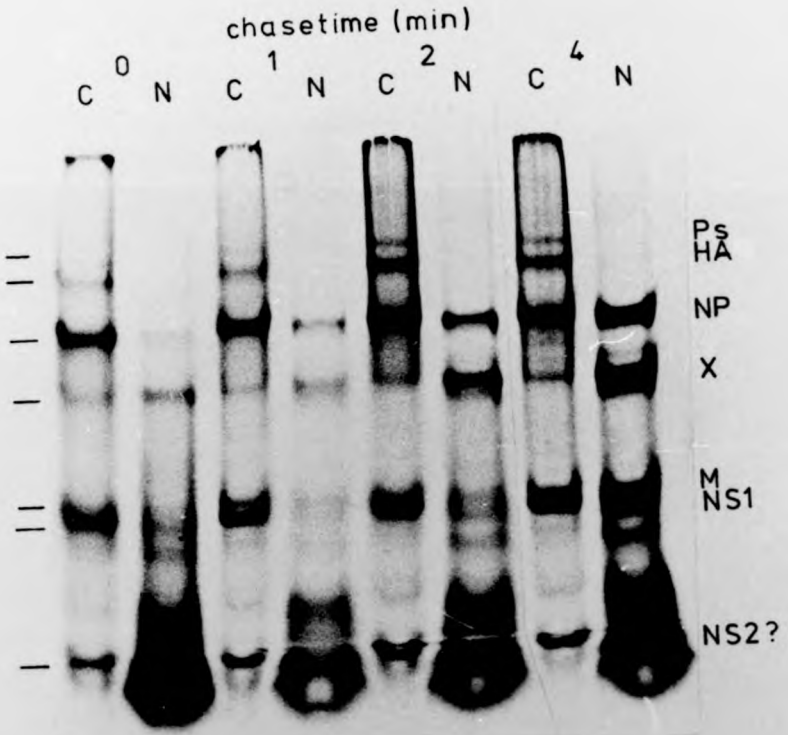
The P proteins synthesized at $1\frac{1}{2}$ and 2 hpi were clearly visible in the cytoplasm but did not appear in the nucleus until chased to 6 hpi. Those synthesized at $2\frac{1}{2}$ and 3 hpi were observed in the nucleus after a 10 min chase, but when labelled at later times the P proteins were found in the cytoplasm.

Fig. 2/13 Distribution of newly synthesized viral proteins between cytoplasm and nucleus. Infected cells were salt-shocked and pulsed for 2 min with [^{35}S] methionine at 5 hpi at 31°. They were chased for up to 4 min as indicated and fractionated by the NML method and analyzed by PAGE (10%/15% phosphate).

C: cytoplasm

N: nucleus

X is the host protein actin.



ral
leus.
d pulsed
hpi at
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ML method
osphate).

(e) HA2

HA2 appeared in the cytoplasmic fractions after a pulse at $3\frac{1}{2}$ and 4 hpi followed by both short and long chases.

In summary this experiment shows that the P proteins, NP and NS1 are synthesized from $1\frac{1}{2}$ hpi but M does not appear until $2\frac{1}{2}$ hpi. HA2 was not observed until after 3 hpi and then in the cytoplasm.

The majority of NP and M became rapidly associated with the nucleus but the P proteins and NS1 associated more slowly. NS1 synthesized up to $2\frac{1}{2}$ hpi was divided about equally between nucleus and cytoplasm; that synthesized at 3 hpi became predominantly nuclear whilst subsequently the newly synthesized NS1 remained cytoplasmic. The P proteins appeared to undergo the same schedule of movement, but this was less convincingly demonstrated. From this qualitative study it was not possible to detect the movement of viral proteins out of the nucleus and we decided that further investigation must be made on a more quantitative basis.

Pulse Chase Experiments: Quantitative Studies on the Location and Movement of Newly Synthesized Viral Proteins

(a) Labelled at 5 hpi at 31° for 2 min and chased for up to 4 min

In the following experiment movements of viral protein were determined shortly after their synthesis (Fig. 2/13). Cultures were salt shocked and starved of methionine at 5 h after infection at 31°. They were pulsed for 2 min with [^{35}S]-methionine and chased for up to 4 min.

As shown in Fig. 2/9 the distribution of total counts between nucleus and cytoplasm varied little over the chase period even though the intensity of viral protein bands differed widely over the same period. About 17% of the total hot TCA precipitable counts were associated with the nuclear fraction. Each track of the autoradiograph was loaded with approximately equal counts; thus the nucleus tracks carried 5 times greater cell equivalent than the cytoplasm tracks.

The nuclear and cytoplasmic fractions exhibited substantial differences in the distribution of viral proteins, both initially and after the chase period. All viral bands increased in both fractions though some cytoplasmic proteins reached a plateau by 4 min. This may represent

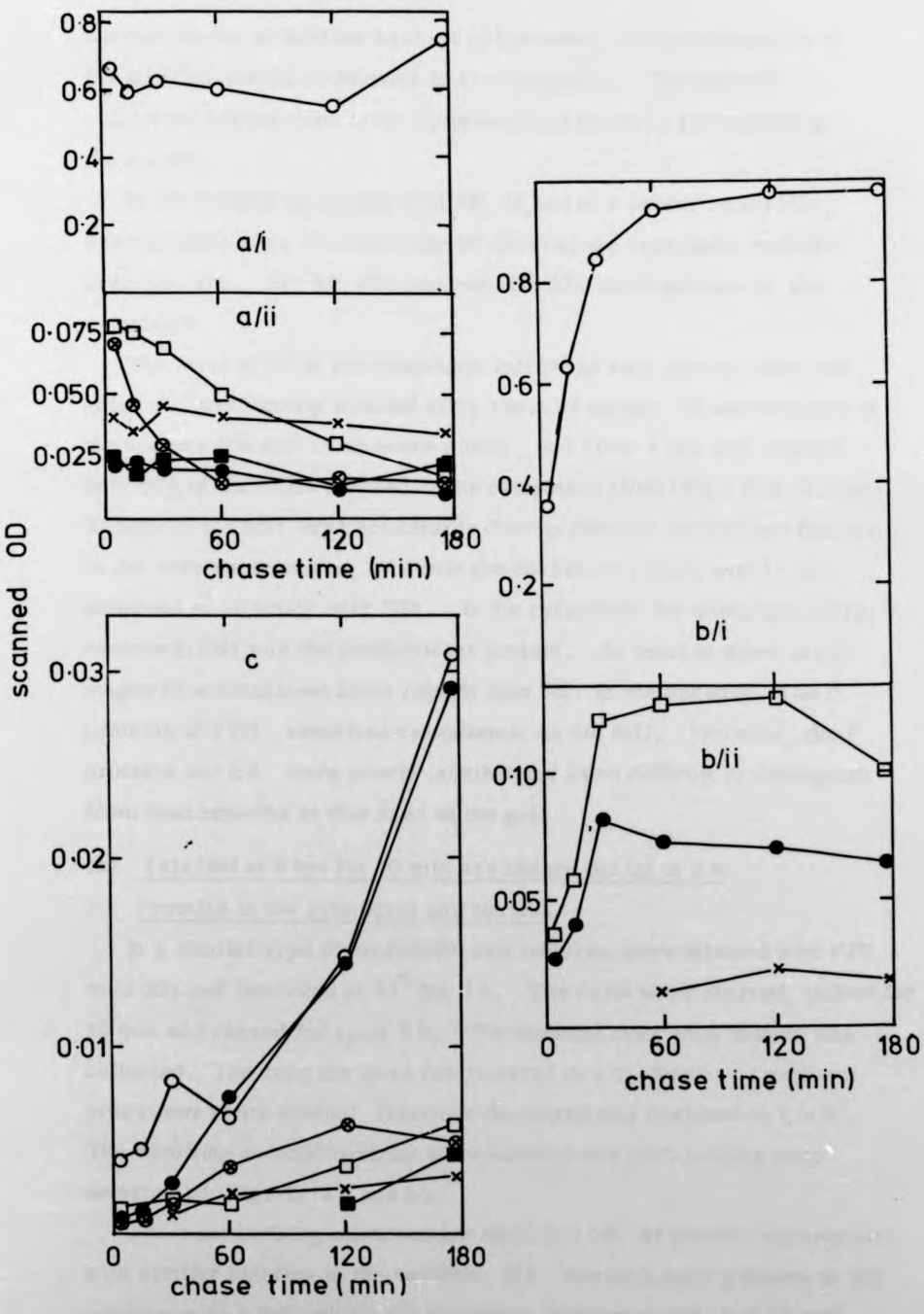


Fig. 2/14 Fate of viral proteins synthesized at 3 hpi.
(a) cytoplasm (b) nucleus (c) tissue culture fluids
○ NP; ● M; □ NS1; ■ HA2; ⊗ HA ; × Ps.

the completion of labelled nascent polypeptides and the exhaustion of the labelled pool of methionine in the cytoplasm. The pool of completed polypeptides in the cytoplasm continues to accumulate in the nucleus.

In the nucleus it is clear that NP, M, and to a lesser extent NS1, were present after the chase period whereas the composite band of P proteins, HA , NP, M, NS1 and possible NS2 were present in the cytoplasm.

The level of NP in the cytoplasm increased very rapidly after the pulse and was heavily labelled after 1 min of chase. NP accumulated in the nuclear fraction much more slowly, and after 4 min had reached only 30% of the value reached in the cytoplasm after only 1 min of chase. Though M and NS1 were not clearly distinguished in the nuclear fraction in the very early stages, by 4 min the doublet is visible with M predominant in intensity over NS1. In the cytoplasm the intensities were reversed; NS1 was the predominant protein. At least in these early stages M accumulated more rapidly than NS1 in the nucleus. The P proteins and HA remained cytoplasmic as did NS2. However, the P proteins and HA were poorly labelled and were difficult to distinguish from host proteins in this area of the gel.

(b) Labelled at 3 hpi for 10 min and chased for up to 3 h

(i) Proteins in the cytoplasm and nucleus

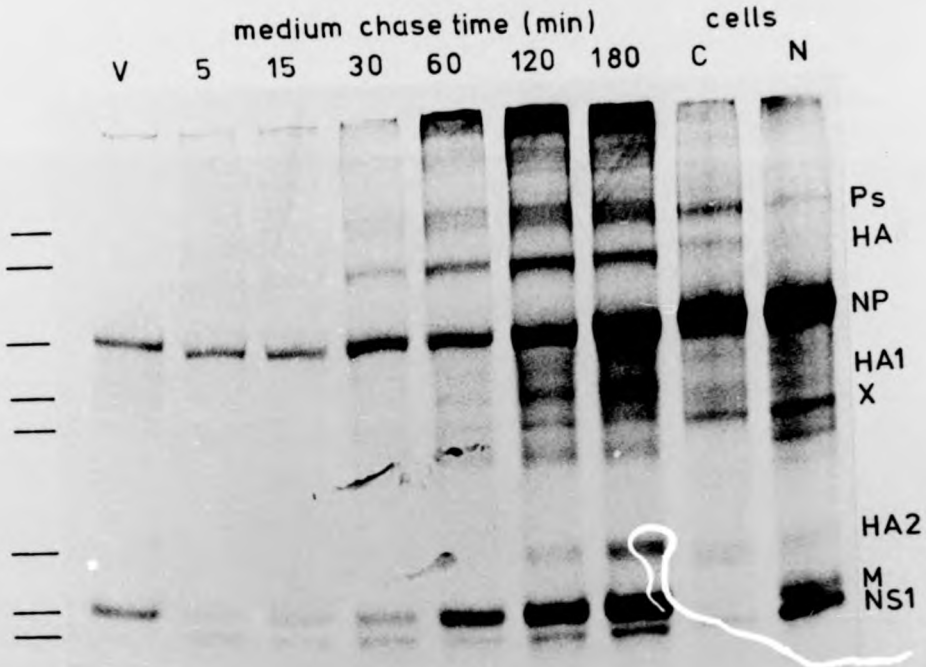
In a similar type of experiment cell cultures were infected with FPV (moi 20) and incubated at 37° for 3 h. The cells were starved, pulsed for 10 min and chased for up to 3 h. The medium over each culture was collected. The cultures were fractionated into nucleus and cytoplasm, precipitated with ethanol, fractions denatured and analyzed by PAGE. The resulting autoradiographs were scanned and peak heights were determined (Fig. 2/14 a and b).

The most striking observations were that NP, M and NS1 accumulated with similar kinetics in the nucleus. HA and HA2 were not seen in the nucleus up to 6 hpi, whilst the P proteins only appeared after 60 min.

Fig. 2/15 Viral proteins released from infected cells into the medium.

Cultures were infected and pulsed with [^{35}S]-methionine for 10 min at 3 hpi. Medium was removed from the cultures after chasing for the times indicated. After 180 min the final cultures were fractionated into cytoplasm (C) and nucleus (N). These tracks contained 12% of the hce of the medium tracks.

X is the host protein actin.


medium chase time (min)
cells
V
5
15
30
60
120
180
C
N
Ps
HA
NP
HA1
X
HA2
M
NS1

In the cytoplasm two groups of proteins could be distinguished according to their movements over the 3 h chase period. NP, Ps, HA2 and M remained at fairly constant levels from synthesis up to 6 hpi, though the concentration of Ps and M fell slightly. On the other hand NS1 and HA showed considerable dilution in the cytoplasmic fraction.

Presumably HA was being cleaved to HA1 and 2 (Lazarowitz et al., 1971; Skehel, 1972), though HA2 did not exhibit a corresponding rise in intensity whilst HA1 was not clearly resolved on this gel system. Alternatively the loss of HA may be due to degradation or the HA species may be unevenly glycosylated so that none of the species are clearly resolved. That the cleavage did occur is seen in the Metrizamide analysis in Section 3.

The level of NS1 in the cytoplasm fell 5-fold, and though the increase in NS1 in the nucleus roughly equalled that lost from the cytoplasm, the kinetics were different. Most of the change in nuclear levels occurred in the first 30 min whilst the loss of NS1 from the cytoplasm was approximately linear throughout the chase period. The increase in nuclear NS1 levels may reflect new synthesis of the protein from the remaining [^{35}S]-methionine pool and its transport to the nucleus.

Labelled at 3 hpi for 10 min and chased for up to 3 h.

(ii) Viral proteins released into the medium

In the medium collected from the cultures immediately prior to fractionation, two groups of proteins were resolved (Fig. 2/14c and 2/15). One, comprising M and NP, exhibited a rapid rise in concentration of approximately 25-fold whilst the other, comprising NS1, HA and P, showed an increase of 3 to 4-fold. HA2 was not catalogued in these groups since it was discernible only after 2 and 3 h of chase and then at very low levels.

This observation is interpreted as showing that initially free viral proteins are released from the cell. The increase in NP and M relative to NS1 through the chase period is seen as the release of labelled virions over and above the continued release of free protein. There may also be a pulse of free NP released within 30 min of the labelling. The

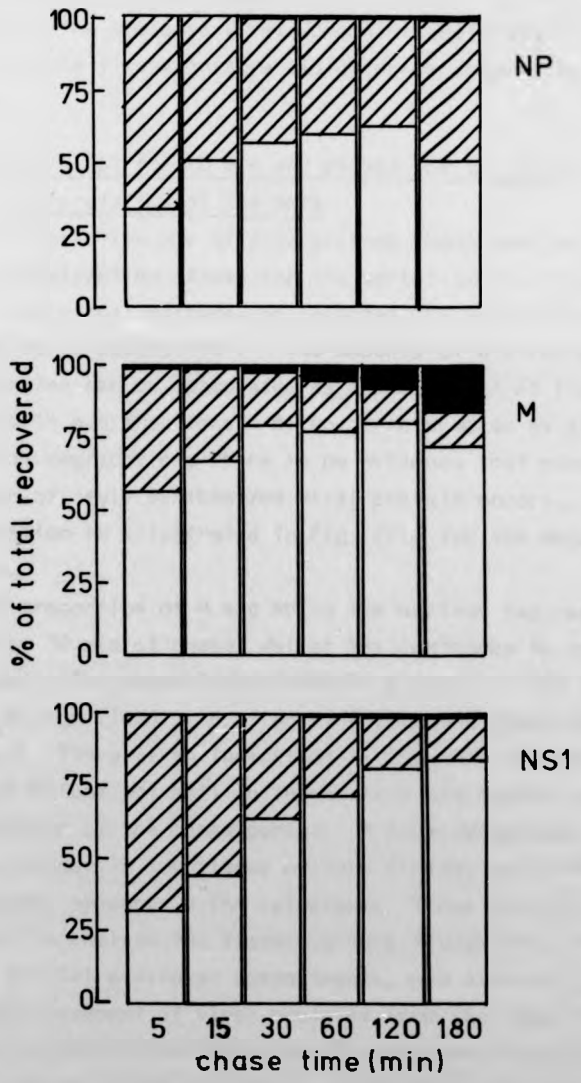


Fig. 2/16 Proportion of major viral proteins in the nucleus, cytoplasm and tissue culture fluids of infected cells during the 3 h following their synthesis at 3 hpi.

Clear: nucleus. Shaded: cytoplasm. Black: TCF.

appearance of HA2 may also result from virion formation whilst the increase in virion Ps could be masked by the continued release of free P proteins. These observations formed the basis of a further analysis of the state of viral components released from infected cells into the tissue culture fluids which forms part 3 of this Section.

Labelled at 3 hpi for 10 min and chased for up to 3 h

(iii) Interpretation of the data

An alternative way of interpreting these data which I have called normalization allows for the variation in the uptake of label between individual cultures of infected CEF cells which I often observed to be substantial. The amounts of a viral protein in each fraction can be calculated as a proportion of the total recovered in all fractions. Although this makes no allowance for non-random degradation, there is no evidence that substantial breakdown of newly synthesized viral protein occurs. This normalization is illustrated in Fig. 2/16 for the major viral proteins.

The proportion of M and NP in the nucleus has reached a plateau by 30 min of chase, whilst NS1 continues to accumulate throughout. The proportion of matrix protein in the tissue culture fluids is significant 1 h after synthesis and rises rapidly to 16% 2 h later. There is an indication in the data that the proportions of NP and M (but not NS1) in the nucleus are beginning to fall in the last hour of the chase period. M from cytoplasm, and possibly nucleus, appears in the tissue culture fluids, whilst NP, lost from the nucleus, appears in the cytoplasm. These data show that it is necessary to analyze the tissue culture fluids for viral proteins as well as the intracellular compartments, and also that, in order to detect any movement of viral proteins from the large pool in the nucleus, normalization techniques to overcome the variation in primary CEF cells are essential. Furthermore, the data indicated that the process of viral protein release appeared to be accelerating and thus a longer chase might reveal a more substantial release of matrix protein and might also show a significant loss of NP into the fluids.

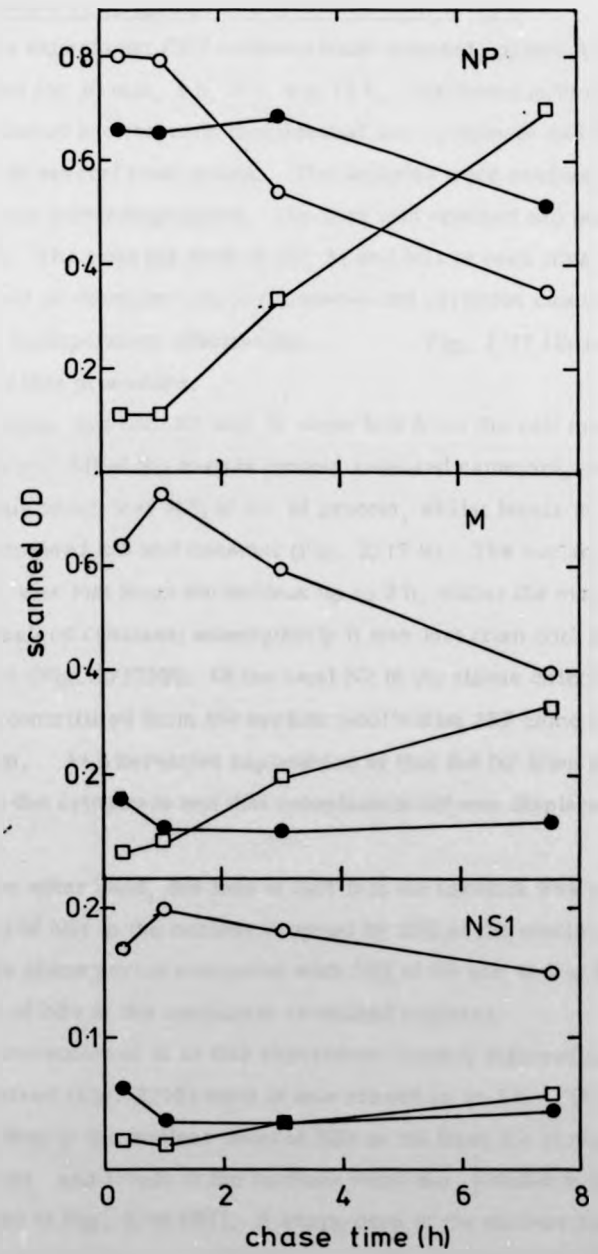


Fig. 2/17 The movement of major viral proteins between nucleus cytoplasm and tissue culture fluids after their synthesis at 3½ hpi.

○ nucleus; ● cytoplasm; □ TCF.

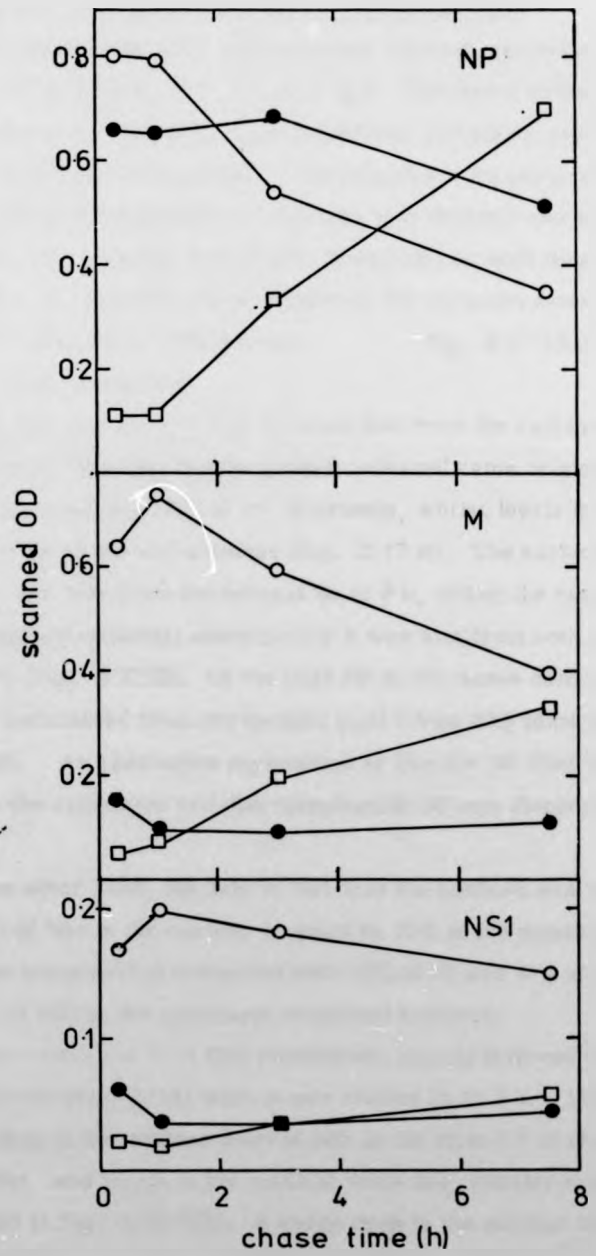


Fig. 2/17 The movement of major viral proteins between nucleus cytoplasm and tissue culture fluids after their synthesis at $3\frac{1}{2}$ hpi.

○ nucleus; ● cytoplasm; □ TCF.

(c) Labelled at $3\frac{1}{2}$ hpi for 10 min and chased for $7\frac{1}{2}$ h

In this experiment CEF cultures were infected, pulsed at $3\frac{1}{2}$ hpi, and chased for 20 min, 1 h, 3 h and $7\frac{1}{2}$ h. The tissue culture fluids were collected and the cells fractionated into cytoplasm and nucleus for each of several time points. The samples were analyzed by PAGE and the gels autoradiographed. The film was scanned and peak heights recorded. The total for each of NP, M and NS1 at each time point was normalized as described above to remove the variation caused by different incorporation efficiencies. Fig. 2/17 illustrates the results of this procedure.

It is clear that both NP and M were lost from the cell monolayer into the medium. All of the matrix protein released came originally from the nucleus which lost 46% of its M protein, whilst levels in the cytoplasm remained low and constant (Fig. 2/17 M). The nucleoprotein, however, was lost from the nucleus up to 3 h, whilst the cytoplasmic level remained constant; subsequently it was lost from both nucleus and cytoplasm (Fig. 2/17NP). Of the total NP in the tissue culture fluids 75% was contributed from the nuclear pool whilst 25% came from the cytoplasm. An alternative explanation is that the NP from the nucleus moved to the cytoplasm and that cytoplasmic NP was displaced into the medium.

On the other hand, the loss of NS1 into the medium was less dramatic. The level of NS1 in the nucleus dropped by 25% of the maximum at the end of the chase period compared with 58% of NP and 46% of M. Like M, the level of NS1 in the cytoplasm remained constant.

The movement of M in this experiment closely followed and extended that observed (Fig. 2/16) when it was chased up to 3 h. There was only a small drop in the nuclear level of NS1 in the first 3 h of chase in this experiment and levels in the medium were low, similar to those illustrated in Fig. 2/16 NSI). A sharp drop in the nuclear levels of NP was observed between 1 and 3 h of chase (Fig. 2/17NPwhilst this was much less marked in Fig. 2/16 i. However, the cells were pulsed at slightly different times ($3\frac{1}{2}$ hpi versus 3 hpi) and Fig. 2/17 probably

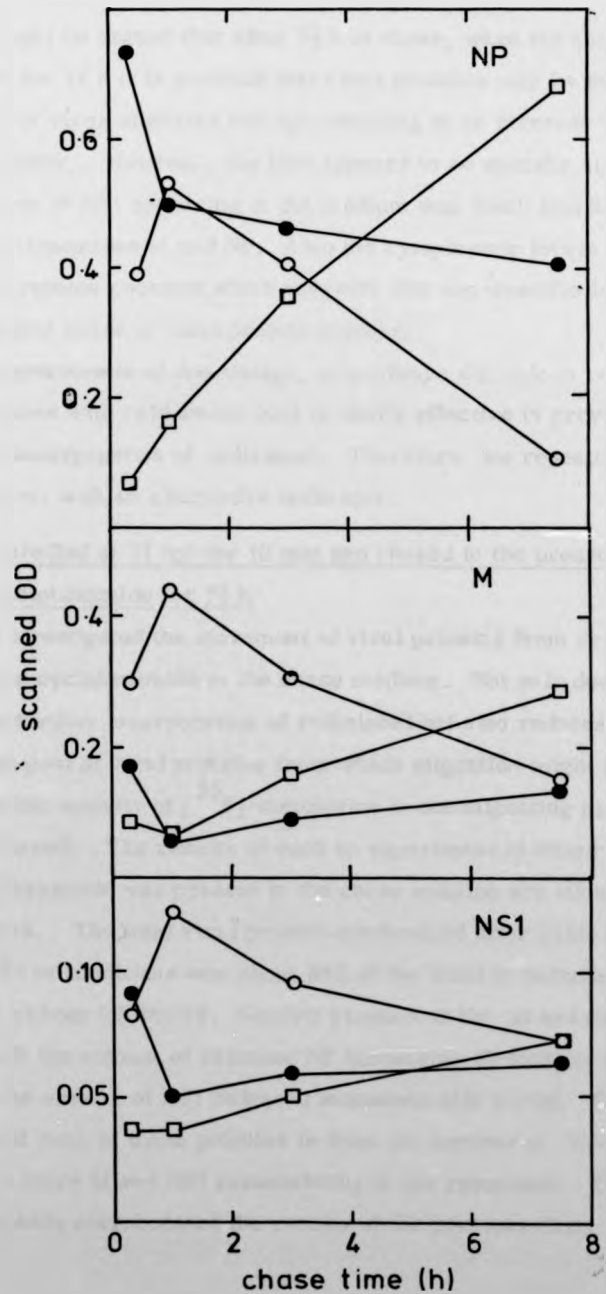


Fig. 2/18 The movement of major viral proteins in infected cells treated with cycloheximide in the chase medium.

○ nucleus; ● cytoplasm; □ TCF.

represents the situation at a later point in the virus multiplication cycle.

It might be argued that after $7\frac{1}{2}$ h of chase, when the cells have been infected for 11 h it is possible that virus proteins may be leaking out as a result of virus mediated damage resulting in an increase in cell permeability. However, the loss appears to be specific since the proportion of NS1 appearing in the medium was much smaller than the virion polypeptides M and NP. Also the cytoplasmic levels of both M and NS1 remain constant, which suggests that non-specific leakage is not a major cause of viral protein release.

In experiments of this design, it is always difficult to be certain that a chase with cold amino acid is really effective in preventing further incorporation of radiolabel. Therefore we repeated this experiment with an alternative technique.

(d) Labelled at $3\frac{1}{2}$ hpi for 10 min and chased in the presence of cycloheximide for $7\frac{1}{2}$ h

We investigated the movement of viral proteins from cell to medium by adding cycloheximide to the chase medium. Not only does this prevent further incorporation of radiolabel but also reduces the size of the total pool of viral proteins from which migration might occur. Thus the specific activity of [^{35}S]-methionine in the migrating proteins would be increased. The results of such an experiment in which 100 µg/ml of cycloheximide was present in the chase solution are illustrated in Fig. 2/18. The total viral protein synthesized after pulse and chase under these conditions was about 80% of the level in cultures pulsed and chased without inhibitors. Similar kinetics of NP, M and NS1 can be seen with the amount of released NP increasing 10-fold, of M 4-fold, whilst the amount of NS1 released increases only 2-fold. The major source of each of these proteins is from the nucleus as before, though there is more M and NS1 accumulating in the cytoplasm. These experiments corroborated the results of the previous Part.

Table 2/2

Activity of neuraminidase in nucleus and cytoplasm

Time after infection	Activity OD_{550}/h/6 x 10^8 hce		Proportion in nucleus
hpi	Cytoplasm	Nucleus	%
* 0.5	15.1	0.5	3
* 2.0	7.5	< 0.2	2
2.5	65.2	3.5	5
5.0	246.0	24.3	9

* These values represent input NA activity and are included for comparison (see also Table 1/1).

Thus we have established, by two different methods, the migration of NP and M proteins from the nucleus and their exit from the cell into the surrounding medium. The analysis of released viral components was extended in the experiments reported in part 3 of this Section.

2B. The Distribution of Newly Synthesized Neuraminidase Between Cytoplasm and Nucleus

Since neuraminidase is not labelled well by [^{35}S]-methionine and is consequently poorly resolved on PAGE, its distribution by activity was determined. It was of great interest to determine the distribution of the very earliest neuraminidase synthesized due to the results obtained by immunofluorescence studies (see this Section 2C) and also the distribution when the majority of the increase in intracellular PFU had taken place (see this Section 3A). In order to determine the earliest point at which newly synthesized, neuraminidase could be detected, the data on input virus (Section 1, 2A), in which cells were assayed for NA at every half hour following infection, was re-employed. Two time points from these experiments are recapitulated in Table 2/2 which also gives the values for NA activity at $2\frac{1}{2}$ and 5 hpi.

The input data showed that the eclipse period was complete at 2 hpi and a substantial rise in total NA activity was expressed at $2\frac{1}{2}$ hpi both over the low value at 2 hpi (8.7-fold) and over the initial input activity assayed 30 min after infection (4.4-fold). This, therefore, represented the earliest time at which newly synthesized NA could be detected. The distribution of activity at this time was heavily in favour of the cytoplasm (95%). At the later time point of 5 hpi no substantial change was observed in distribution (91% cytoplasmic) though the amount of neuraminidase had increased to 400% of its level at $2\frac{1}{2}$ hpi.

Fig. 2/19 Immunofluorescence studies of NP in infected cells

(a) 2 hpi

(b) 3 hpi

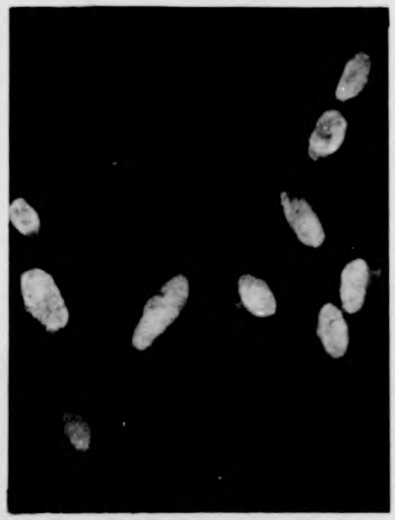
(c) 4 hpi

(d) 5 hpi

(e) 7 hpi

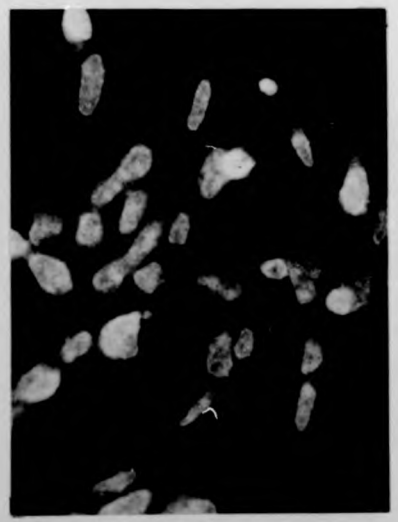
in

a



b

c



d

e

Fig. 2/20 Immunofluorescence studies of NA in infected cells

(a) 1 hpi

(b) $1\frac{1}{2}$ hpi

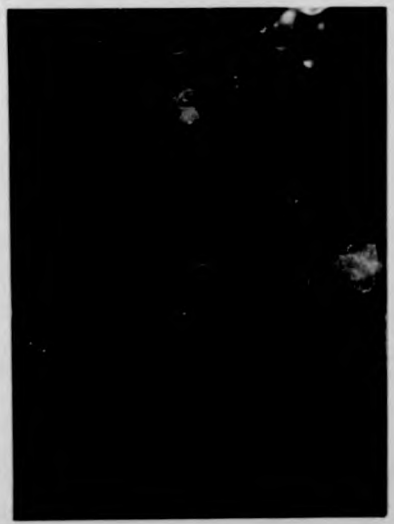
(c) 2 hpi

(d) $2\frac{1}{2}$ hpi

(e) 5 hpi

a

b



c

d

e

2C. The Movement of Viral Antigens Between Cytoplasm and Nucleus

Antisera to specific viral components was prepared (Kelly and Dimmock, 1974) and the technique of indirect immunofluorescence used to visualize viral antigens within cells at various times after infection.

(a) NP antigen

The antigen was observed in the nucleus at 2 hpi (Fig. 2/19a). About 50% of the cells showed this bright specific nuclear fluorescence (arrows) whilst the remainder exhibited only background levels. At 3 hpi (Fig. 2/19b) the nuclear fluorescence was much brighter and in over 90% of the cells. One hour later (Fig. 2/19c) the cytoplasmic level of fluorescence had increased. In some nuclei a bright annulus of fluorescence was observed peripheral to the nuclei (arrow) and within the nucleus the level of fluorescenceseemed to be lower. By 5 hpi the level of NP antigen in the cytoplasm had increased further (Fig. 2/19d) whilst by 7 hpi (Fig. 2/19e) the whole cell was fluorescing sometimes with a concentration around the periphery (arrow), and the nucleus could not often be distinguished from the cytoplasm by its fluorescent intensity.

These results show that NP antigen accumulated in the nucleus of infected cells at 2-3 hpi. Between 4 and 5 hpi the cytoplasmic level increased which may result from further synthesis of NP or from pre-existing antigen in the nucleus migrating back into the cytoplasm as evidenced by the peripheral staining of some nuclei. By 7 hpi the antigen appeared to be present in the whole cell with a concentration at the cell periphery.

(b) NA antigen

At 1 hpi the cells generally exhibited background fluorescence though tiny specks of fluorescence were often observed in the cytoplasm of the cells (arrow Fig. 2/20a). None were present in the nucleus. At 1½ hpi (Fig. 2/20b) there was a small increase in cytoplasmic fluorescence which appeared to be spread diffusely, whilst the overall level of the

nucleus was lower. Within the nucleus, however, bright specks of fluorescence could be seen, and the nucleus was surrounded by a ring of brightness (arrow). Half an hour later, this perinuclear ring of fluorescence was plainly visible (Fig. 2/20c) though the level of stain in cytoplasm and nucleus appeared equal. At $2\frac{1}{2}$ hpi the NA antigen was concentrated within the nucleus which fluoresced intensely (arrow Fig. 2/20d). About 70-80% of the cells exhibited this nuclear fluorescence. At 5 hpi fluorescence levels were much higher and most cells fluoresced with equal intensity in the cytoplasm and nucleus.

This result showed similarities with the appearance and movement of NP antigen and although the serum had been extensively absorbed to remove any NP antigen, a check on its specificity was performed. A parallel experiment was set up in which cells were infected with FP/BEL (Hav1 .N1) and FP/R (Hav1.Neq1). At no time up to 4 hpi was fluorescent antigen observed in the nucleus of FP/BEL infected cells though it appeared in FP/R infected cells at 2-$2\frac{1}{2}$ hpi. When FP/BEL infected cells were subjected to anti-NP serum, the characteristic nuclear fluorescence pattern of NP was produced from 3 hpi. Thus the anti-NA serum did not contain contaminating anti-NP antibodies or other antibodies reacting with common antigens such as M or possibly P proteins, and the nuclear accumulation of NA antigen was a genuine event.

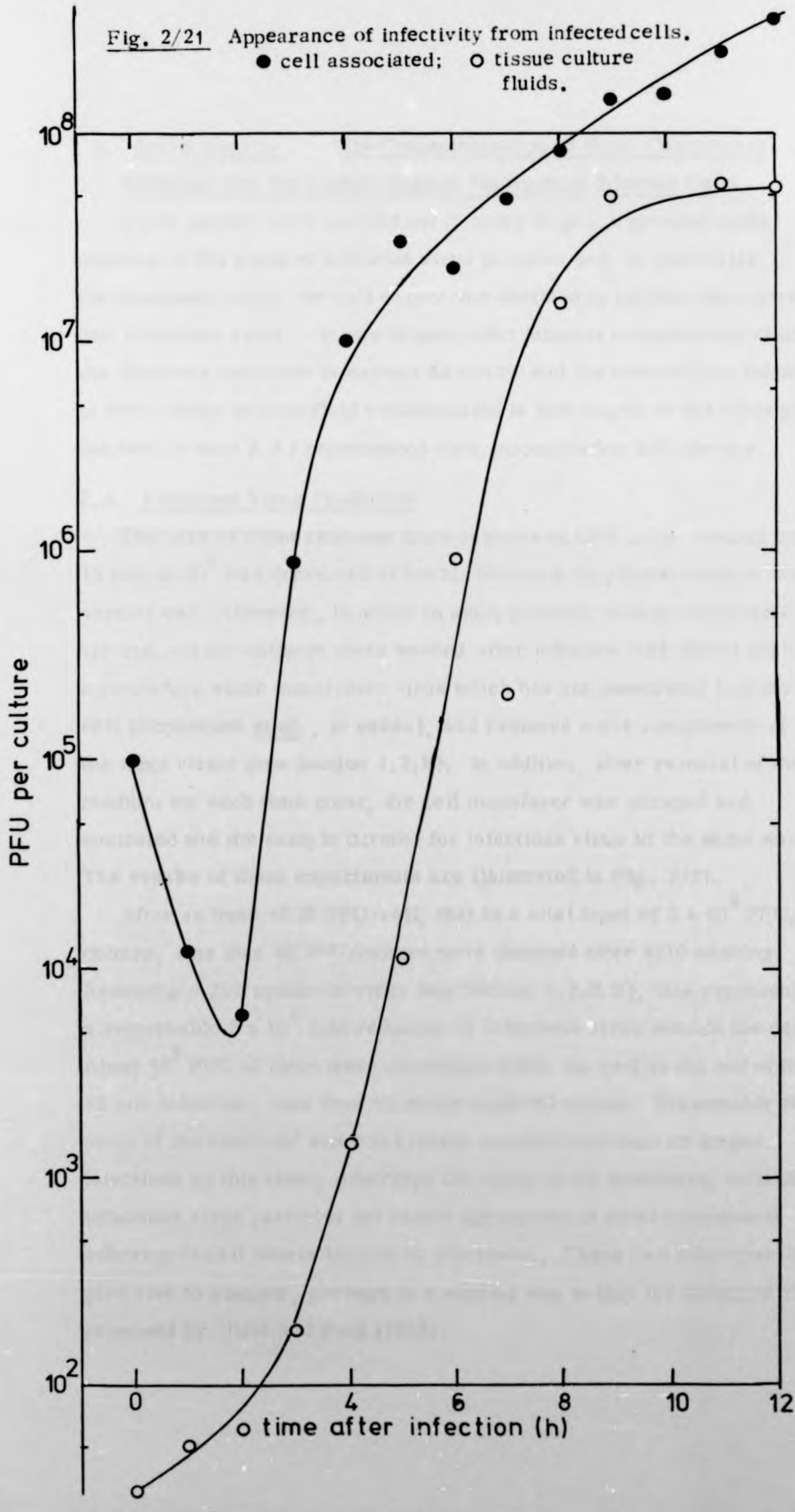


Fig. 2/21 Appearance of infectivity from infected cells. ● cell associated; ○ tissue culture fluids.

3. Extracellular The Characterization of Viral Components Released into the Tissue Culture Fluids from Infected Cells

These studies were carried out in order to gain a greater understanding of the roles of influenza virus proteins and, in particular, the movement within the cell of proteins destined to become incorporated into infectious virus. It was of particular interest to determine whether the observed reduction in nuclear M and NP and the concomitant increase in their tissue culture fluid concentration in late stages of the virus growth cycle (as seen in part 2.A) represented their incorporation into virions.

3.A Infectious Virus Production

The titre of virus released from cultures of CEF cells infected for 15 min at 20^{o} was measured at hourly intervals by plaque assay in the normal way. However, in order to study genuine, newly synthesized virions, all the cultures were washed after infection with PBS at pH 3, a procedure which inactivates virus which has not penetrated into the cell (Stephenson et al., in press), and removes some components of the input virion (see Section 1.2.B). In addition, after removal of the medium for each time point, the cell monolayer was scraped and sonicated and the sample titrated for infectious virus in the same way. The results of these experiments are illustrated in Fig. 2/21.

After an input of 30 PFU/cell, that is a total input of 3×10^{8} PFU/culture, less than 40 PFU/culture were detected after acid washing. Assuming a 25% uptake of virus (see Section 1.2.B.b), this represents a remarkable 5×10^{6} fold reduction in infectious virus outside the cell. About 10^{5} PFU of virus were detectable within the cell at the end of the 15 min infection, less than 1% of the expected uptake. Presumably then, much of the absorbed virus is already uncoated and thus no longer infectious by this time; otherwise the assay is not measuring individual infectious virus particles but rather aggregates of viral components adhering to cell debris formed on sonication. These can subsequently give rise to plaques, perhaps in a similar way to that for defective virions proposed by Hirst and Pons (1973).

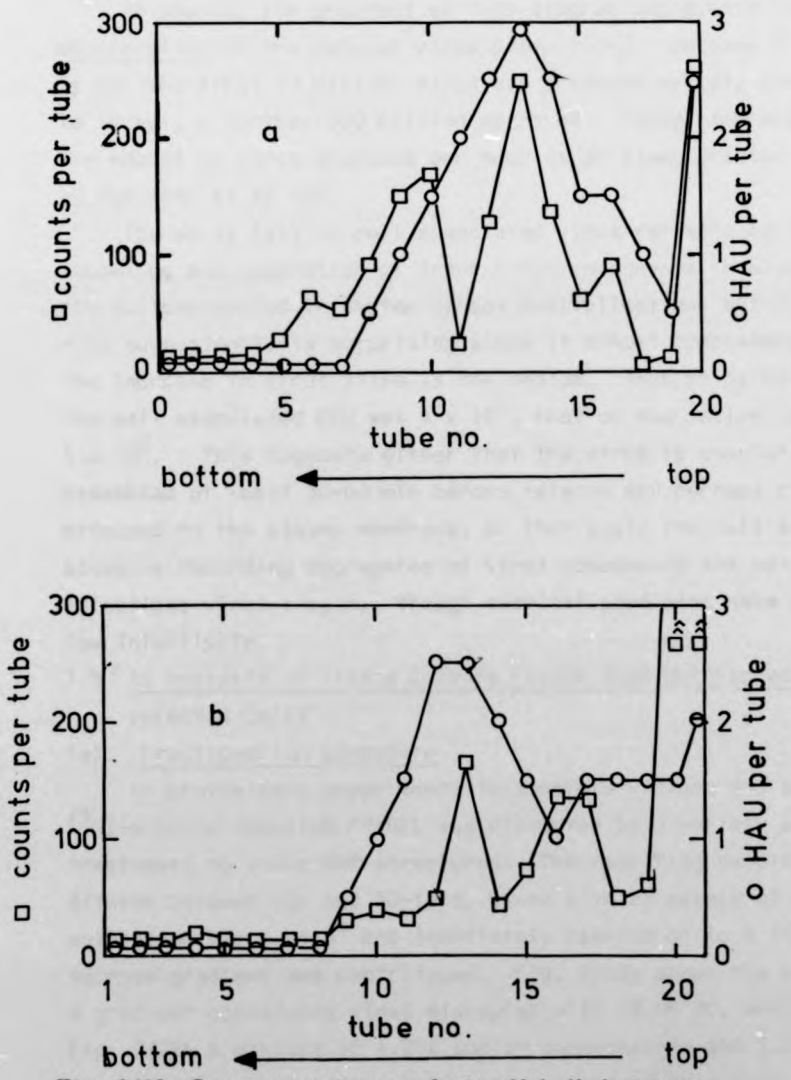


Fig. 2/22 Sucrose gradient analysis of labelled virions:

(a) disrupted with 1% NP 40

(b) disrupted with 1.25% sodium deoxycholate and 2.5% NP 40.

Cell-associated infections virus first fell 30-fold and then rose rapidly 1000-fold between 2 and 3½ hpi post-infection. This rapid rise was followed by a more modest rate of increase to 12 hpi of a further 50-fold. The infections virus recorded in the medium rose sharply from 3½ to 7 hpi, increasing 4 $\log_{10}$ units in this period. It levelled off at 9-10 hpi at a final value of 5×10^7 PFU/culture.

Of course, the gradient of this diagram represents the acceleration of the rate of virus production. Between 2 and 3½ hpi the first 10 million virus are produced whilst, from 3½ to 12 hpi, a further 500 million appeared. Thus, for example, the amount of virus produced per hour is 30 times greater at 10 hpi than at 3½ hpi.

The early fall in cell associated virus reflects continuing uncoating and separation of input virus components (analagous to the eclipse period in bacteriophage multiplication) but the sharp rise subsequently is surprising since it almost completely precedes the increase in virus titre in the medium. Thus at 3½ hpi, whilst the cell associated PFU was 4×10^6, that of the medium is only 5×10^2. This suggests either that the virus is completely assembled at least 30-60 min before release and perhaps remains attached to the plasma membrane, or that again the cell associated assay is recording aggregates of viral components and not complete infectious virus progeny, though subviral particles have a very low infectivity.

3.B An Analysis of Tissue Culture Fluids from Uninfected and Infected Cells

(a) Fractionation procedure

In preliminary experiments to separate virions and substructures. [^{3}H]-uridine labelled FP/BEL was disrupted by a variety of treatments to yield RNP structures. The resulting samples were diluted between 10- and 50-fold, mixed with an excess of purified unlabelled virus at 4° and immediately layered on to a 10-45% sucrose gradient and centrifuged. Fig. 2/22a shows the profile of a gradient containing virus disrupted with 1% NP 40, whilst in Fig. 2/22b a mixture of 1.25% sodium deoxycholate and 2.5% NP 40 was used. In both cases whole virus was found in a peak at fraction 13 from the radiolabelled sample co-running with the haemagglutinin marker of the purified virus. Occasionally another peak was observed which ran in front of the virus peak (Fig. 2/22a, fractions 9 and 10). This was thought to be a virus aggregate caused by

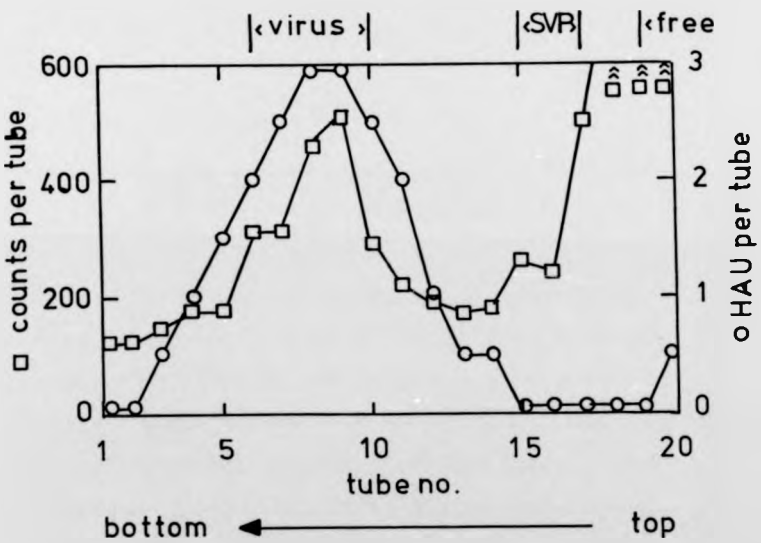


Fig. 2/23 Typical sucrose gradient profiles of tissue culture fluids from infected labelled cells.

Fig. 2.24 Virus proteins present in the medium from infected cells.

Infected cells were pulsed for 30 min at 2.5 hpi with [^{35}S] methionine and the medium was collected at each hour, being replaced by fresh medium. The aliquots of tissue culture fluids were processed for 10% phosphate PAGE.
C and N respresent the cytoplasmic and nuclear fractions of the culture after 12 hpi at half the hce of the aliquots of medium.

X is the host protein actin.

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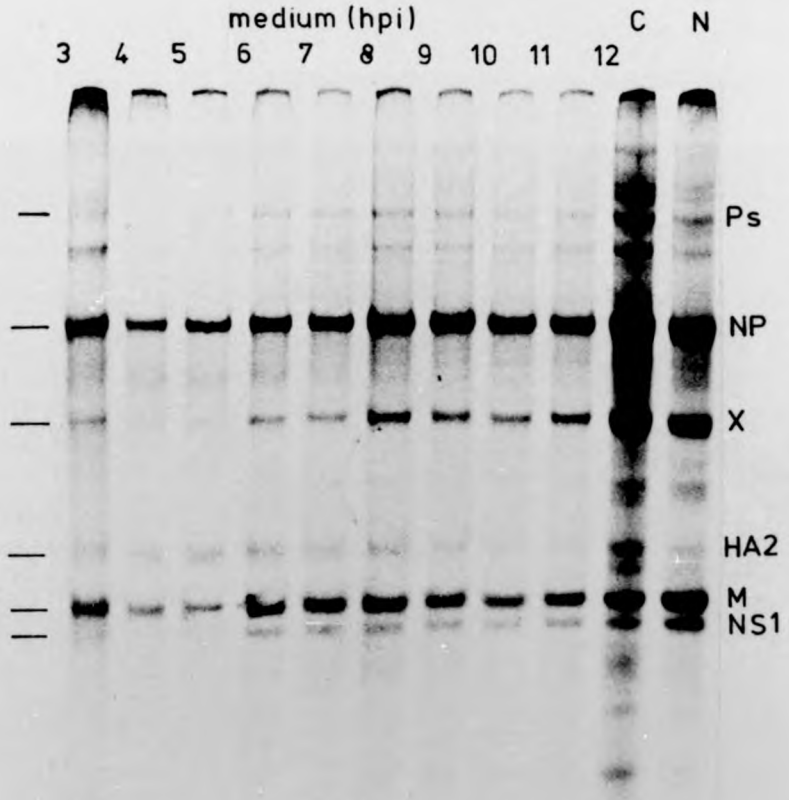


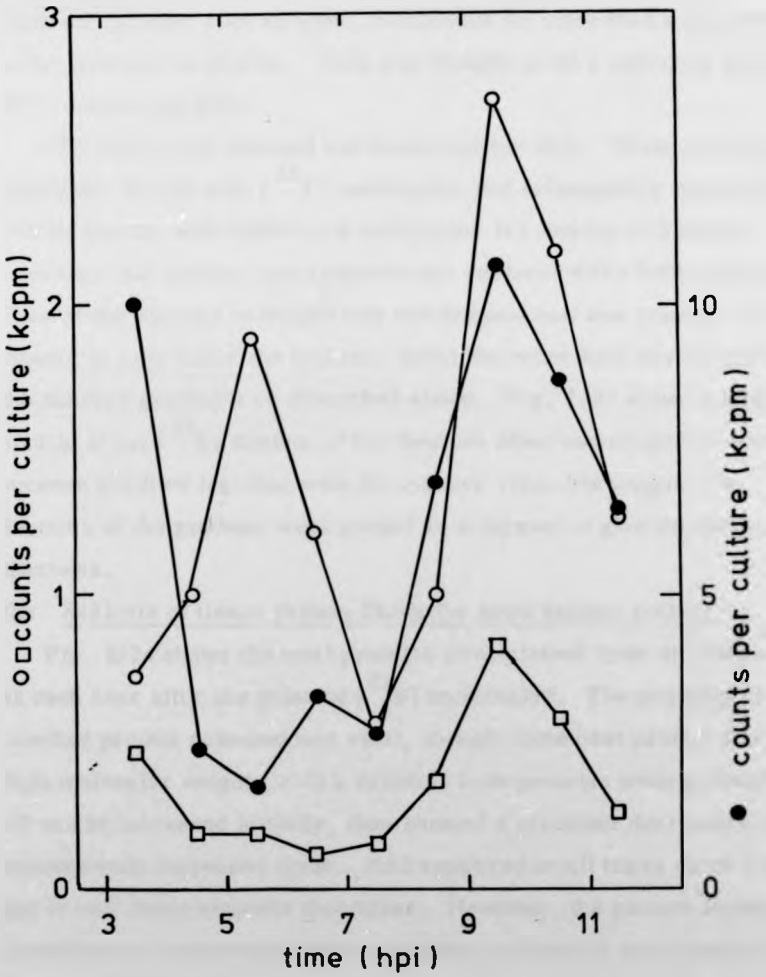
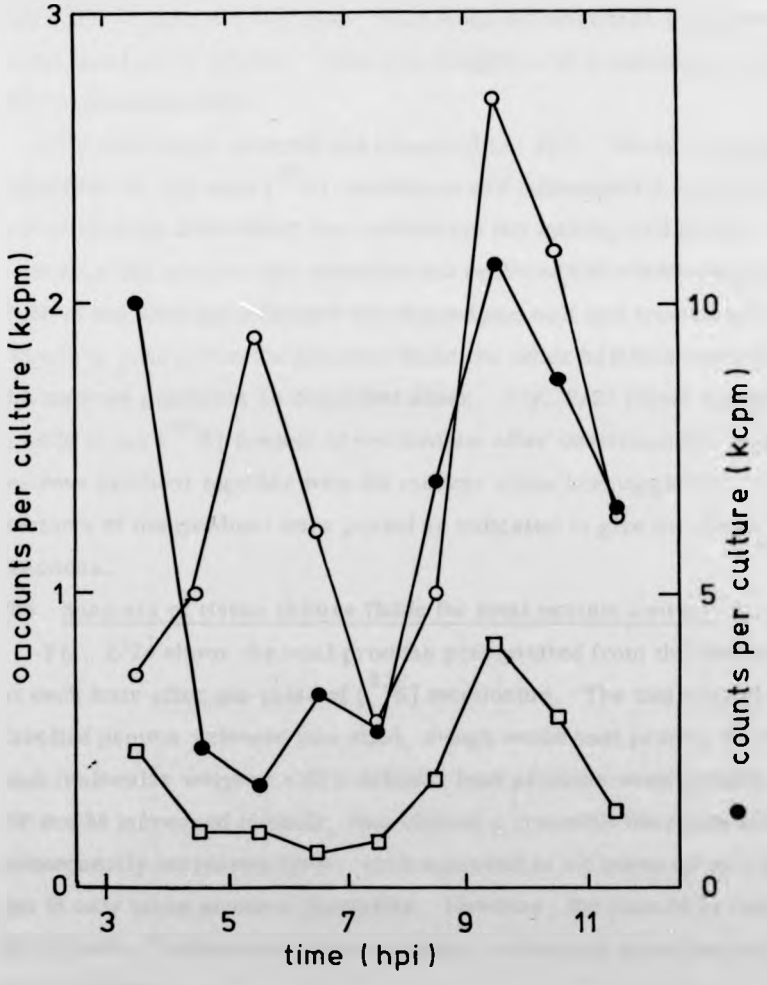


Fig. 2/25 Content of TCF from infected cells between 3 and 12 hpi. Infected cells were pulsed with [^{35}S] methionine for 30 min at 2,5 hpi. Culture fluids were harvested each hour and replaced with fresh medium.

The samples were analyzed on sucrose gradients into ● free, □ SVP's, and o virus, precipitated out and the radioactivity measured.



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The samples were analyzed on sucrose gradients into ● free, □ SVP's, and o virus, precipitated out and the radioactivity measured.

the detergent treatment. Cytochrome c and other free proteins were never observed below the top two fractions. Between the virus and free protein accumulations there can be seen a small peak, sometimes no more than a shoulder, in the radioactive profile. This was thought to be a subvirion particle (SVP) containing RNA.

CEF cells were infected and incubated for $2\frac{1}{2}$ h. These were then pulsed for 30 min with [^{35}S]-methionine and subsequently chased in growth medium with added cold methionine but lacking calf serum. At each hour the medium was removed and replaced with a fresh aliquot. Each of the aliquots collected was divided, and half was treated with ethanol to precipitate the proteins whilst the other half was centrifuged on the sucrose gradients as described above. Fig. 2/23 shows a typical profile of the [^{35}S] content of the medium after centrifugation on a sucrose gradient together with the marker virus haemagglutinin. Sections of the gradient were pooled as indicated to give the three fractions.

(b) Analysis of tissue culture fluids for total protein content

Fig. 2/24 shows the total proteins precipitated from the medium at each hour after the pulse of [^{35}S] methionine. The majority of the labelled protein released was viral, though some host protein X (actin) and high molecular weight (> 60 k daltons) host proteins were present. NP and M increased initially, then showed a transient decrease and subsequently increased again. HA2 appeared at all times up to 9 hpi but in only trace amounts thereafter. However, the picture is confused by the lack of separation between virions, sub virion particles and free proteins.

Fig. 2/25 illustrates the profiles of total counts incorporated into virions, SVP's and free proteins. Labelled virions were found at 5-7 hpi and 9-11 hpi, whereas free protein and SVP's increased at around 9-11 hpi only. Both the free protein and SVP's are present at a high level in the first hour after synthesis and subsequently decline. They do not show any peak coincident with the release of virions at 5-7 hpi. It should be noted that the release of free protein, as denoted by the right hand axis, is about five-fold greater than that of labelled protein in virions (left hand axis).

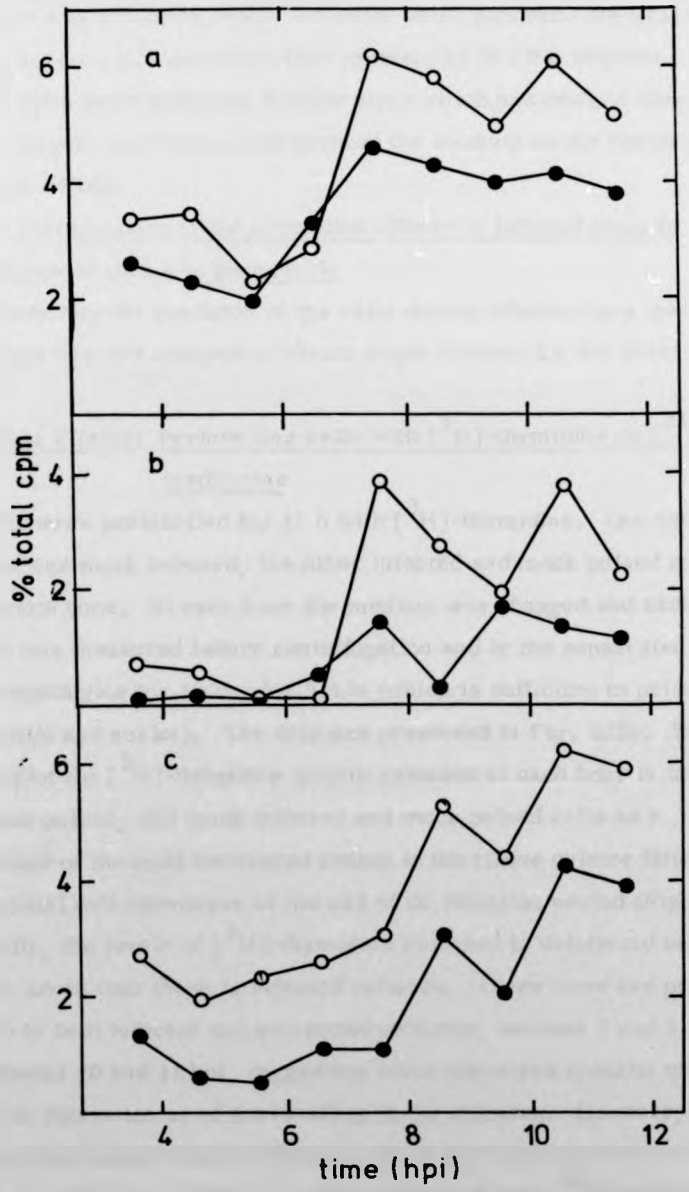


Fig. 2/26 Appearance of prelabels in the tissue culture fluids of infected and uninfected cells. Method: see Fig. 2/25.
(a)(b): [^{3}H] thymidine (c) [^{35}S] methionine

(a) total counts as % of total incorporated: O infected; ● uninfected.
(b) counts precipitated by low speed centrifugation: O infected ● uninfected
(c) O total counts as % of total incorporated; ● counts precipitated by low speed centrifugation.

This biphasic release of virus containing labelled protein synthesized between $2\frac{1}{2}$ and 3 hpi suggested that (i) a second cycle of virus production was occurring within the cells which produced the first batch and was utilizing pre-existing virus proteins or (ii) the original infected cells were releasing further virus which had become absorbed to their plasma membranes and entered the medium as the result of cytopathic effects.

(c) An investigation of the cytopathic effects in infected cells by analysis of tissue culture fluids

A study into the condition of the cells during infection was made to investigate whether cytopathic effects might account for the observed data.

Cytopathic Effects: Prelabelling cells with [^{3}H]-thymidine or [^{35}S]-methionine

Cells were prelabelled for 15 h with [^{3}H]-thymidine. One set of cultures was mock infected, the other infected and mock pulsed at the appropriate time. At each hour the medium was changed and radioactivity was measured before centrifugation and in the supernatant after centrifuging for 1000 g for 5 min (which is sufficient to pellet whole cells and nuclei). The data are presented in Fig. 2/26. The graphs plot the [^{3}H]-thymidine counts released at each hour in infected and mock pulsed, and mock infected and mock pulsed cells as a percentage of the total recovered counts in the tissue culture fluids, and the residual cell monolayer at the end of the infection period (Fig. 2/26a, b). Generally, the levels of [^{3}H]-thymidine released in uninfected cells were slightly lower than those in infected cultures. There were two peaks of release in both infected and uninfected cultures, between 7 and $8\frac{1}{2}$ hpi and between 10 and 11 hpi, suggesting some non-virus specific effects of serum starvation or of the handling of the cultures. However, those effects were exacerbated by infection which increased the precipitable counts by 175% (Fig. 2/26b). Cells prelabelled with [^{35}S]-methionine, infected and mock pulsed, showed a similar distribution (Fig. 2/26c).

Cytopathic Effects: Analysis of tissue culture fluids by Coulter counting

Aliquots of tissue culture fluids were analysed with a Coulter counter. There were insignificant counts in the range of large cellular debris and whole cells, but debris of up to 10 μm was detected. Size was estimated by correlation with latex beads of known diameter. A comparison of the debris released from infected and uninfected cultures (Fig. 2/27) showed that the release from infected cultures varied between 60 and 220% greater than that of uninfected cells. The release was low at first and exhibited the greatest difference between infected and uninfected cells at 7-8 hpi.

Cytopathic Effects: The appearance of host protein 'X' in the tissue culture fluids from infected cells

A host protein commonly present in infected cell extracts (MW, 45,000 daltons) labelled X (Skehel, 1972) and thought to be actin, was observed in the free /proteins released from infected cells. The amount of this protein released from infected cells is plotted in Fig. 2/28. There was a small peak at 3-4 hpi and at 6-7 hpi, and a much larger one at 8-9 hpi, and levels remain similarly high to the end of the period. A total of 29% of actin (X) was released.

Cytopathic Effects: The release of virus nonstructural protein (NSI) into the tissue culture fluids

The release of NSI dropped from a high level in the first hour of chase to rise again to a peak at 7-8 hpi, after which it was released at about 5%/h for the rest of the chase period. 42% of NSI was released in all.

Cytopathic Effects: A comparison of the release of 'viral' with that of 'host cell' components

There was a small rise in pelletable host protein at 3-4 hpi after the mock pulse and a high level of released thymidine counts of which very few were pelletable. This did not correspond in scale to the large output of free virus proteins observed in Fig. 2/25. There was very little pelletable thymidine or host protein released at the time of the

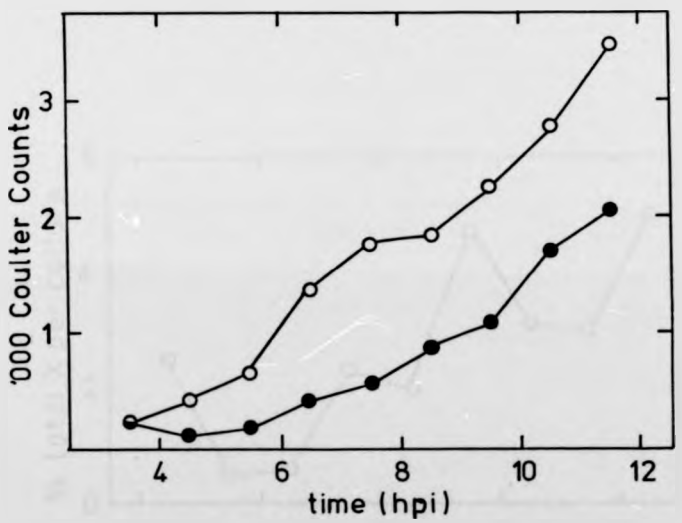


Fig. 2/27 Appearance of cell debris in tissue culture fluids from infected and uninfected cells.
Method: see Fig. 2/25.

o infected cells

● uninfected cells

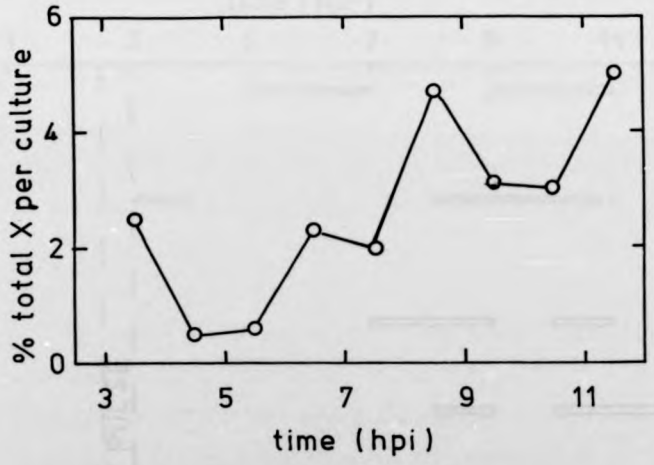


Fig. 2/28 Appearance of host protein X (actin) in the tissue culture fluids from infected cells.
Method: see Fig. 2/25. Samples were analyzed by 10% phosphate PAGE.

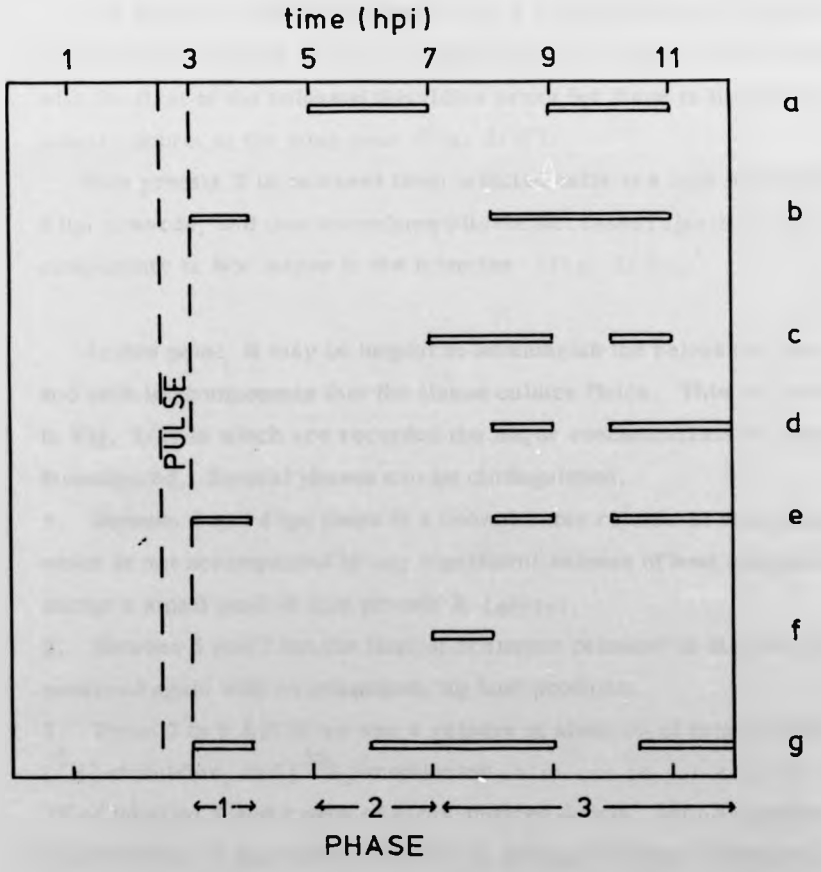
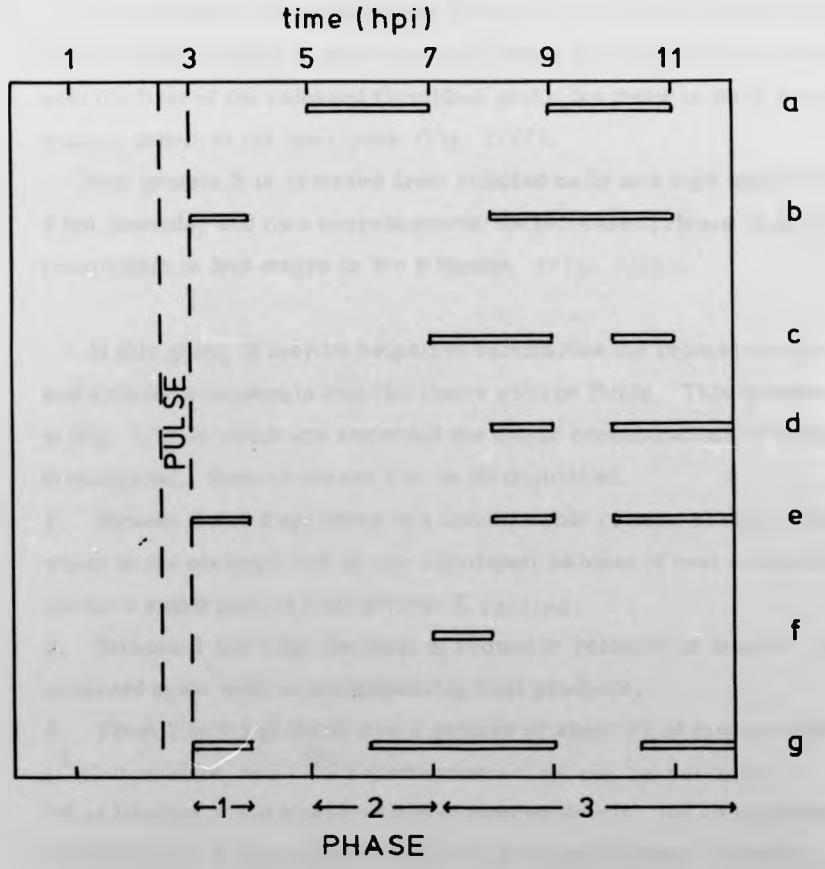


Fig. 2/29 Major concentrations of viral and cellular components released into TCF.

(a) virions; (b) free viral proteins and SVP's;

(c) precipitable prelabel thymidine; (d) precipitated prelabel methionine; (e) host protein X; (f) virus induced debris; (actin)

(g) NSI.



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(a) virions; (b) free viral proteins and SVP's;

(c) precipitable prelabel thymidine; (d) precipitated prelabel methionine; (e) host protein X (actin); (f) virus induced debris;

(g) NSI.

first virion peak at 5-6 hpi (compare Fig. 2/25 with Fig. 2/26b, c), whilst no correlation was observed between the first peak of thymidine release at 7-8½ hpi and release of virion components. However, thymidine and NSI were released at the same time. The second peak of pelletable thymidine and methionine from infected and mock pulsed cells at 10-11 hpi overlapped the second peak containing virions, SVP's and free viral protein at 9-11 hpi.

The data from Coulter counting show a large increase of 218% over the uninfected culture of virus-induced debris at 7-8 hpi which coincides with the first of the released thymidine peaks, but there is little virus induced debris at the later peak (Fig. 2/27).

Host protein X is released from infected cells at a high level from 8 hpi onwards, and thus correlates with the increased release of all host components at late stages in the infection (Fig. 2/28).

At this point, it may be helpful to summarize the release of viral and cellular components into the tissue culture fluids. This is illustrated in Fig. 2/29 in which are recorded the major concentrations of components investigated. Several phases can be distinguished.

1. Between 3 and 4 hpi there is a considerable release of viral components which is not accompanied by any significant release of host components except a small peak of host protein X (actin).
2. Between 5 and 7 hpi the first of two major releases of labelled virions occurred again with no accompanying host products.
3. From 7 to 9 hpi there was a release of about 6% of total prelabel [^{3}H]-thymidine, and [^{35}S]-methionine which can be pelleted at 1000 g, about 7% of labelled X and a peak of virus-induced debris, but no significant concentration of any virion component, although NSI was released.
4. From 9 to 11 hpi there was a further release of virions, a peak of SVP and free viral proteins. Also released were a further 10% of total pre-label [^{3}H]-thymidine of which 6% was in a form pelletable at 1000 g. Host protein was released which consisted of X, at least in part, and the levels of released NSI were about 5%/h.

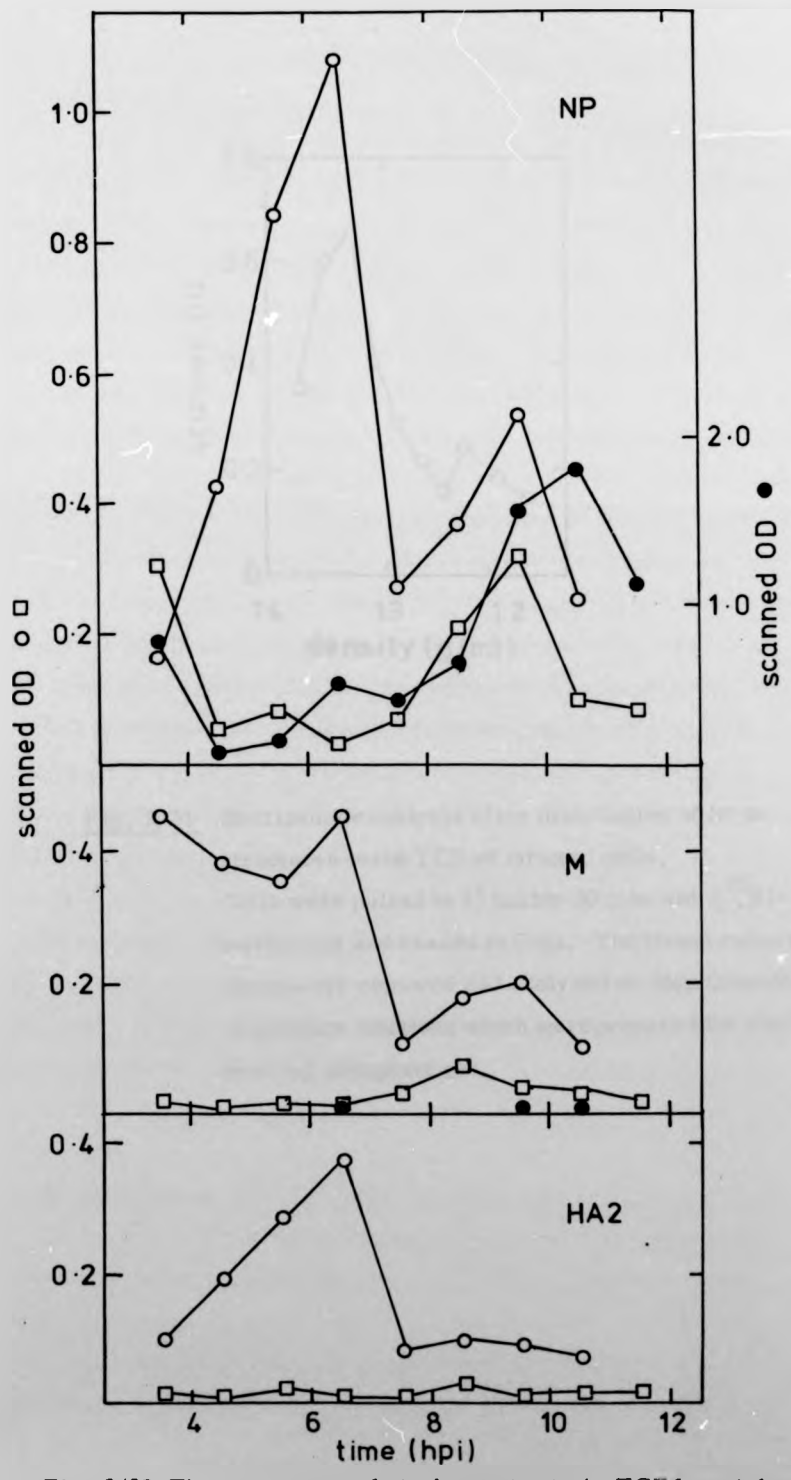

Fig. 2/30 The appearance of viral proteins in the TCF from infected cells. Method: see Fig. 2/28. o virus; ● free; □ SVP.

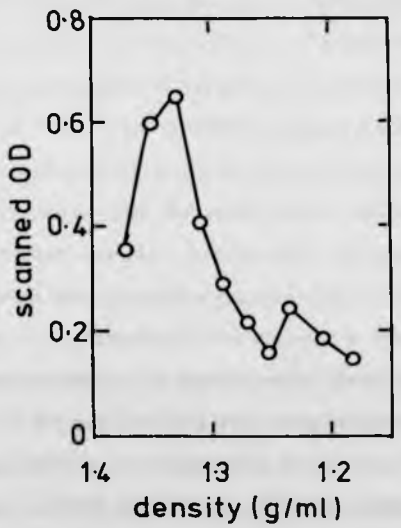

Fig. 2/31 Metrizamide analysis of the distribution of NP in structures in the TCF of infected cells.
Cells were pulsed at 2½ hpi for 30 min with [^{35}S]-methionine and chased to 7 hpi. The tissue culture fluids were removed and analyzed on Metrizamide to produce fractions which were prepared for PAGE on a 10% phosphate gel.

(d) The distribution of viral proteins between virions, SVP's and in the soluble fraction

The analysis of the distribution of the viral proteins between the three fractions is shown in Fig. 2/30. Both HA2 and NP labelled between $2\frac{1}{2}$ and 3 hpi appear in virions in steadily increasing amounts up to a peak at 5-7 hpi (Fig. 2/30NP, HA2)but for different reasons. The delay in incorporation of NP into virions may be due to its role in forming RNP structures with the viral RNA's whilst the haemagglutinin must be cleaved from the HA precursor. Contrast the behaviour of these proteins with that of matrix protein (Fig. 2/30M) which appeared in relatively constant amounts in the virions in samples from 3-7 hpi. Thus this protein seems to be incorporated directly into the virion. It is noticeable that the pool of HA2 was completely utilized in the first peak of virus production and there was little extra released in the second burst at 9-10 hpi, whilst both M and NP were present at 9-10 hpi at about half their concentration in the first peak of virus release.

It is surprising that, of the viral proteins resolved, only NP was found free (Fig. 2/30NP)and most of this appeared in the first hour after synthesis and as a rather diffuse peak over the last 3 h of incubation. Traces of M could be observed at 9-11 hpi and also at 6-7 hpi, but no free HA2 and only traces of NS1, too small to quantify, were released.

There were differences in the distribution of the proteins in the SVP and in the other fractions. M and HA2 were resolved (albeit at a low level) together with traces of NS1 and a large amount of NP in a peak at 8-10 hpi. There was no peak of any labelled viral protein in the SVP fraction at the time when the first release of virus occurred at 5-7 hpi.

In an attempt to further characterize the nature of the structure in which viral proteins were released into the medium, the medium from cells pulsed between $2\frac{1}{2}$-3 hpi and chased to 7 hpi was layered on to 37% Metrizamide and centrifuged to equilibrium. Due to poor labelling, only NP was clearly observed and the distribution of this across the gradient is illustrated in Fig. 2/31. The majority of NP

present appears at the density of free protein (1.325 g/ml) in agreement with the previous data, and there is no indication of the presence of RNP structures (at 1.255 g/ml). However, a small peak of NP was observed at the density of virions (1.23 g/ml).

4. DISCUSSION

In this section I have clearly demonstrated the migration of NP out of the nucleus by two different methods, and have correlated this with the movement of NP antigen. I have shown that matrix protein accumulates in the nucleus together with NP and NSI, and also moves out again whilst NSI remains predominantly nuclear. I have shown a bimodal distribution of viral proteins, synthesized early in infection in released virions and assessed the contribution of cytopathic effects to this phenomenon. I have demonstrated the release of free viral NP shortly after synthesis. These results show that M is incorporated into virions immediately after synthesis, whilst there is a slow build-up of NP and HA2 incorporation confirming the observations of Hay (1974).

4.A Temporal Expression of Viral Proteins at 37°

FP/Rostock infected CEF cells exhibited a temporal expression of virus protein synthesis at 37° in agreement with Skehel (1972). Ps, NP and NSI were detectable by pulse-labelling at 1½ hpi, whilst M appeared 30 to 60 min later and HA2 appeared at 3½ hpi. NA could be detected by its activity at 2-2½ hpi and was observed by immuno-fluorescence techniques at 2 hpi. This division of early (Ps, NP, NSI) and late (M, HA, NA) proteins corresponds to the two groups of proteins whose synthesis was distinguished by different classes of drugs (Minor and Dimmock, 1975, 1977). It is also similar to the division of viral proteins according to their site of synthesis on free or membrane bound ribosomes (McGeoch et al., 1976).

However, Meier-Ewert and Compans (1974) have described a constant rate of synthesis for all detectable viral proteins in a WSN infected BHK cell system except that the synthesis of M increased at

later times. Lamb and Choppin (Lamb, R.A. and Choppin, P.W. at Negative Strand Virus Symposium, Cambridge, 1977) found the synthesis of M detectable at $\frac{1}{2}$ hpi in WSN infected BHK cells, and could find no differences in the rate of synthesis of any proteins throughout the infection period. These authors do find a different sequence of protein synthesis in different host cells, and it appears that the variation may depend on the concentration of a host protein as described in the introduction.

4.B Growth of Virus at 31°

At 31°, the course of viral protein synthesis and, in particular, the change from excess NSI to predominantly M synthesis between 4 and 6 hpi, confirms the observations of Skehel (1973). However, in contrast to Skehel, I found that moi 10 or greater was essential for this time course. The infection of cells with low moi at 4° and subsequent incubation at 31° led to unreliable virus growth. Incorporation of [^{35}S]-methionine into viral polypeptides was sometimes not observed for 6 h. Although the occurrence of CEF cells which are resistant to attachment and penetration has been described (Stephenson et al., in press) this does not completely explain the variation since, on warming to 31°, the inhibition of penetration should be overcome. Perhaps the virus infectivity becomes inactivated quickly if it cannot penetrate as has been documented for pollovirus (Joklik and Darnell, 1961) in which a non-infectious particle lacking VP4 is eluted in the course of a normal infection. The degree of this elution depends in part on the physical state of the cell. If this is also true for influenza, it might explain the variability of the 4° infection experiments.

4.C Comparison of 31° and 37° Growth Cycles

A comparison of the timescale of infection at 31° with 37° shows that the appearance of intracellular HA activity is delayed about 1 h, and the peak of infectious virus production is less and 2 h later. NP antigen appears at about 4-5 hpi, 2 h later than at 37°. However, only

about 5% of the cells at 31° showed an increase in cytoplasmic NP levels after a further 2 h whilst the majority showed this 5-9 h later than at 37°. Possibly the small population of cells which show the NP antigen accumulating again in the cytoplasm at 7-8 hpi may be the cells producing infectious virus.

4.D Nuclear Accumulation of Viral Proteins

NP, M and NSI proteins were observed in the nucleus by pulse-labelling and NP antigen in the nucleus by immunofluorescence. Radiolabelled HA2 was always found in the cytoplasm. NA activity was found in the cytoplasm but NA antigen was found first in a peri-nuclear then a nuclear location. NP antigen has been located in the nucleus (Breitenfeld and Schafer, 1957; Maeno and Kilbourne, 1970; Kelly and Dimmock, 1974) and a non-structural antigen, believed to be NSI, was reported to be concentrated in the nucleolus (Dimmock, 1969). By pulse-labelling, various workers have located NP and NSI in the nuclear fraction (Taylor et al., 1969, 1970; Lazarowitz et al., 1971, Krug and Etkind, 1973; Krug and Soeiro, 1975).

Taylor et al. (1969, 1970) found VP3, which they thought to be matrix protein, in the nucleus, but Lazarowitz et al. (1971) reinterpreted this as being the non-structural protein which is very similar in size to M, as they did not find M in the nucleus in their studies. However, Lazarowitz et al. found very little M in whole cells at the time chosen, and hence it is doubtful whether it would be resolved in the nuclear fraction. Similar problems and the difficulty of resolving M from NSI may account for the failure of Krug and coworkers (Krug and Etkind, 1973; Krug and Soeiro, 1975) to detect M in the nucleus.

Alternatively, the immunological studies of Oxford and Schild (1975) in which they detected M antigen in the nuclei of some cells and always with accompanying cytoplasmic fluorescence, may suggest that the nuclear appearance of M is a variable phenomenon. The antiserum of Oxford and Schild was prepared against SDS-treated matrix protein, however, and may not have antibodies to all native M antigenic

determinants. In contrast, Gregoriades (1973) using an acidified chloroform methanol extraction, located M in the nucleus. Though she recently claimed that this method extracted both M and NSI (Gregoriades, A. at Negative Strand Virus Symposium Cambridge, 1977), in our hands, using a gel system which clearly distinguished M and NSI the method is specific for M (data not presented). The work of Hay and Skehel (1975) also showed that matrix protein, synthesized at 5 hpi in CEF cells, accumulated in the nucleus with NP and NSI.

There was apparent discord between the immunological data which show nuclear fluorescence and assays of NA activity in cytoplasmic and nuclear fractions which indicate that 90-95% of the newly synthesized activity is cytoplasmic. Whilst there is some justification for suggesting that antigenic differences might exist between the native protein and a labelled SDS-disrupted protein on PAGE, the NA antigen and active NA enzyme must be very similar. Although it is possible to envisage activity without antigenicity and vice versa, due to the separate antigenic and enzymic sites of the molecule, it does seem unlikely that the 5-10% of activity in the nucleus is the only enzyme in antigenic configuration. The appearance of NA fluorescence peripheral to the nucleus has been described in previous immunological studies (Breitenfeld and Schafer, 1957; Maeno and Kilbourne, 1970; Kelly and Dimmock, 1974).

It is possible then that the apparent nuclear fluorescence observed in our study consists of the cytoplasm overlaying the nucleus and that the NA antigen does not penetrate the nuclear membrane. However, in none of the other studies has such an observation been reported whilst, if this explanation is correct, it would be commonly observed. Both Breitenfeld and Schafer (1957) and Maeno and Kilbourne (1970) presented data on widely separated time points in the infection cycle and, if there was a sequence of perinuclear, -nuclear, -whole cell fluoro-escence, they might have missed the nuclear phase.

If this were so, the simplest explanation of the inconsistencies of our experiments is that NA exists in two forms, both active, but only one in the antigenic state exhibited in the virion, against which the antiserum was prepared. This might be due to incomplete glycosylation or attachment to lipid, but both these possibilities would seem to favour antigen being predominant in the cytoplasm rather than vice versa. Thus it is not resolved whether, and if so in what form, NA is present in the nucleus at 2-2½ hpi.

The perinuclear ring of fluorescence, which is a common finding in all the immunofluorescence studies, may simply be due to the synthesis of NA on rough ER immediately adjacent to the nuclei, but the concentration so close to the nucleus and its apolar nature (Breitenfeld and Schafer, 1957; Maeno and Kilbourne, 1970) suggest a more specific association. Marcus et al. (1965) have demonstrated the presence of sialic acid residues in the nuclear membrane, and it is possible that the newly-synthesized neuraminidase is attached to these. If so, its function there is entirely unknown,

but speculatively it could be involved in mediating the passage of viral RNP's out of the nucleus just as the input NA might be involved in effecting the entry of input vRNP's.

4.E. Movement of Viral Proteins out of the Nucleus: NP

I have shown movement of NP out of the nucleus of infected CEF cells by pulse-chase experiments. This could be detected at 4½ hpi and continued until 11hpi when 50% of the total radiolabelled NP had migrated. I have confirmed this observation by chasing in the presence of cycloheximide and under these conditions a greater proportion (80%) of NP moved from the nucleus. This enhancement is attributed to cycloheximide reinforcing the cold chase by stopping protein synthesis and probably more importantly inhibiting further production of unlabelled NP which would otherwise dilute the radioactively labelled NP. I have demonstrated by immunofluorescence that, in our system, NP antigen accumulated first in the

nucleus and then appeared in the cytoplasm in increasing amounts, sometimes with a concomitant decrease in nuclear fluorescence. This can be interpreted as the movement of the NP antigen from the nucleus which is simultaneous with an increase in the amount of NP moving from its site of synthesis in the cytoplasm to the nucleus.

The failure to observe a decrease in nuclear fluorescence as a result of the migration of the NP antigen out of the nucleus may be caused by the rising amount of NP synthesized in the cytoplasm moving into the nucleus. The increase in cytoplasmic levels of fluorescence is unlikely to be due to newly synthesized NP polypeptides alone since the antiserum does not react with denatured RNP. At late times it is possible, however, that newly synthesized NP may be rendered antigenic in the cytoplasm without recourse to the nucleus.

There is good correlation between the movement of NP measured by radiolabelled protein in the presence and absence of cycloheximide. Although the use of any inhibitor of cell function is open to the criticism that the normal processes of the cell are distorted, the correlation of the native migration with that observed with the inhibitor and the validation of the rationale suggest that the cycloheximide data represent the normal migration. The effects of cycloheximide on this process have attracted little study. Taylor et al. (1970) found that the inhibitor did not prevent the accumulation of NP and M/NSI in the nucleus. Cycloheximide, if added early in infection, restricts RNA synthesis to primary transcription (Bean and Simpson, 1973; Lamb and Choppin, 1976) and inhibits the synthesis of vRNA (Scholtissek and Rott, 1970; Pons, 1973), but it is not known at what point this inhibition no longer affects the production of virions.

The movement of radiolabelled NP from the nucleus from 5 hpi onwards correlates with the migration of the NP antigen which is first detectable at 4 hpi and more marked at 5 hpi, and these movements coincide with the peak of labelled proteins in virions from 5-7 hpi and

the rapid rise in infectious virus in the tissue culture fluids of 1000-fold between 4½ and 7 hpi. This is discussed at greater length below. Thus, by two independent means, we have shown that NP moves out of the nucleus.

The migration of NP antigen (the g-antigen) into the cytoplasm was described by Breitenfeld and Schafer (1957) for FPV infected CEF cells. They found nuclear fluorescence between 3 and 5 hpi, cytoplasmic fluorescence increased from 8 to 10 hpi, whilst the whole cell fluoresced at 14 hpi. The reason for this very long timescale is unknown. It may be concerned with the characteristics of growth of the cell line used in those experiments and the difference between these results and more recent data may be accounted for by the improvement in cell culture media and techniques. Maeno and Kilbourne (1970), using 1/5C/4 human tissue culture cells, found that after nuclear fluorescence at 3 hpi the levels of antigen in the cytoplasm had risen by 7 hpi and, at a later time (17 hpi), the whole cell fluoresced. A move from nucleus to cytoplasm at 3.5 hpi was reported by Kelly and Dimmock (1974). The most straightforward interpretation of this observation is that NP antigen migrates back from the nucleus into the cytoplasm, although it could also be explained by the loss of antigenicity of NP in the nucleus and its acquisition by NP in the cytoplasm.

The observation that pulse-labelled NP can be chased back out of the nucleus has not been reported previously. Indeed, Hay and Skehel (1975) reported that NP continued to be accumulated in the nucleus until 9-10 hpi, and that no loss was observed. Krug and Etkind (1973) also failed to detect any migration of NP from the nucleus. There are a number of reasons for this apparent disparity.

1. It is becoming clear that there are differences between infected host cells in the amount and time of appearance of viral proteins and in the proportion of viral components used in viral processes, including productive virion assembly. In some cell lines NP may be made in excess, and the amount which migrates may be too small to detect.
2. In most cultured cells there appears to be a rapid and substantial accumulation of NP in the nucleus. It is much more

difficult to observe a small decrease in this large amount than the corresponding several-fold increase in the tissue culture fluids, especially in the early stages, and unless the process is followed to late times it might be overlooked.

3. These studies have shown that crossover kinetics of NP migration are observed between nucleus and tissue culture fluid, but not between nucleus and cytoplasm, i.e. that a drop in the level of nuclear NP corresponds to a rise in the level of NP in the medium. Although one cannot exclude that NP leaving the nucleus results in an equivalent amount of cytoplasmic NP moving into the TCF, I prefer the interpretation that NP passes through the cytoplasm without accumulating there. Previous studies have not examined the virus proteins present in the tissue culture fluids on a quantitative basis.

4. There may be quantitative or qualitative differences in transport of NP at different times after infection. I was careful to choose an early time in the growth cycle ($2\frac{1}{2}$-$3\frac{1}{2}$ hpi) which preceded the major release of infectious virus (4-$7\frac{1}{2}$ hpi) and yet labelled both early and late proteins. This maximized the probability of labelling those viral proteins most likely to be transported and incorporated into virions.

However, Hay and Skehel (1975) observed no migration of NP from the nucleus even though they were using FPV in CEF cells like those of the present study, but they did label at 5 hpi when in this system the first rise in cell associated virus was virtually complete. I have not followed the migration of NP which was labelled as late as 5 hpi, but it is possible that the profile of virus protein transport is more confused at this later timepoint due to increasing asynchrony of the virus growth cycle. The relatively large amount of newly synthesized virus protein at later times may mask the productive movement of the small amount of potential viral proteins.

The movement of viral proteins from the nucleus: M, NS1

The movement of matrix protein out of the nucleus occurred rather more quickly than NP as illustrated in Fig. 2/16 in which 16% of M appeared in the medium at 6 hpi compared with 2% of NP, but this early lead was not apparent in the longer chase experiments. 50% of the nuclear M was released into the medium over a $7\frac{1}{2}$ hpi chase, whilst

70% of that chased in the presence of cycloheximide was found in the tissue culture fluids. This resulted in a 4-6-fold rise in the levels of M in the tissue culture fluids. In contrast, NSI was lost from the nuclei into the medium, but less rapidly. There was a 25% drop in nuclear NSI after a $7\frac{1}{2}$ h chase and levels in the medium rose 2-3-fold. As in the case of NP above, it is possible that at least some of the M and NSI in the TCF originated from the cytoplasm and was displaced by proteins coming from the nucleus.

It might also be argued that the viral proteins are accumulating in the medium due to cytopathic effects on the cells and that the proposed transport from nuclei to tissue culture fluids is simply due to the proteins leaking out of dying cells or in cells which have become detached into the medium. This aspect is discussed at some depth below in relation to the components present in the tissue culture fluids, but there are several lines of evidence which suggest that transport from the nucleus is specific, at least until the late stages of infection.

1. The loss of NSI from the nucleus and its accumulation in the medium is less than that of M and NP. If this loss was due to the cytopathic effect, the proportional losses should be similar.
2. The cytoplasmic levels of M, NP and NSI are generally stable. A cytopathic effect might be expected to reduce cytoplasmic levels fast and certainly as much as nuclear amounts. A slight fall is observed in cytoplasmic levels of NP, but that of M and NSI rises slightly in the same circumstances.
3. There is an indication from Fig. 2.16 that NP accumulates in the cytoplasm before appearing in the medium early in the release process. It seems probable that once virus release has begun that transport from the nucleus might be co-ordinated and thus there would be no accumulation of viral proteins from the nucleus in the cytoplasm.

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4.F The Appearance of Viral and Host Components in the Tissue Culture Fluids: Infectious Virus

The majority of infectious virus is released into the medium, under our conditions, between $3\frac{1}{2}$ and 9 hpi. However, cell-associated virus (CAV) reaches a high level prior to this increase and exhibits a biphasic profile (Fig 2/21). The rate of accumulation of CAV is about 2 $\log_{10}$ per hour between 2 and 4 hpi, but it decreases to 8-fold per hour between 5 and 12 hpi. CAV is not expected from a virus which buds from the plasma membrane. Why then does this phenomenon occur ?

CAV might arise by aggregation of viral components which are in close proximity at assembly sites near the plasma membrane. Sonication is known to induce membranes to vesiculate and to form large aggregates, and these actions might be involved in the artificial production of infectivity. Although, with a stretch of the imagination, this can be envisaged as a very occasional probability, the observation that most of the CAV appears at early stages suggest that virus arises by natural means. SVP's are unlikely to contribute significantly as these are known to have a very low infectivity. The second possibility, somewhat akin to the first, is that virus is delayed whilst in the process of budding and that the sonication induces the premature completion of this step. However, evidence of several workers indicates that M is incorporated immediately prior to virion release, hence artefactually created virions would lack M and probably be non-infectious. Despite the objections, these theories of incomplete assembly are attractive in offering an explanation for the continued rise in CAV even after release of virions into the medium has levelled off. If the assembly of virus was inhibited by a shortage of components at later times in infection and some form of 'aggregate association' was possible, the observed rise in CAV could be explained.

The third and most likely explanation is that CAV is infectious virus which has been assembled and released but has readsorbed to the monolayer. This could occur between 2 and 4 hpi when there is apparently little virus release into the medium. Between 4 and 10 hpi virus is released into

the medium whilst the rate of rise of CAV is slowed. Released virus might either be newly assembled and released directly, or CAV might be a precursor to the released virus. Speculatively, a combination might be the case in which the budding of newly assembled virus through membrane with a localized concentration of reabsorbed virions might result in release of either the new or absorbed virus. But this explanation of reabsorbed virus does not account for the continued accumulation of cell-associated virus, even after the release of infectious virus into the medium is complete. It is possible that this represents a second cycle of infection which might lead to a further release of infectivity into the tissue culture fluids providing the cells remained viable.

The appearance of viral and host components in the tissue culture fluid: labelled components

Virus proteins synthesized between $2\frac{1}{2}$ and 3 hpi were incorporated into virions released at 5-7 hpi and 9-11 hpi, and comprised about 20% of the total labelled protein released. Free protein was released in the first hour after synthesis, and between 8 and 11 hpi. The putative SVP fraction will be discussed in detail below, but broadly its distribution followed that of the free protein.

The study of cytopathic effects in infected cells showed that though there was a loss of 30-45% of infected host cell and viral components into the medium over this period, a significant loss was also observed in uninfected cells. The three phases defined in the results (see Fig. 2.29) are best considered separately. In the first hour after the pulse (phase 1) there was a considerable release of free labelled protein comprising about 20% of the total released. Apart from a small amount of host protein X, this was predominantly viral protein. Analysis of the tissue culture fluids showed no significant release of host cell components in either uninfected or infected cells, thus making it unlikely that damage to the cell monolayer caused during the washing and pulsing procedures was responsible for this release. The ejection of some free viral proteins from infected cells shortly after their synthesis thus seems to be a natural phenomenon. The composition of this release is discussed below.

In phase two, between 5 and 7 hpi, there was a peak of labelled proteins in released virions. At this time the release of host cell components, as measured by all methods, was low and there was no significant release of free labelled viral protein. This shows that the maximum incorporation of proteins into virions occurs about 2-4 h after their synthesis. The utilization of individual viral proteins is detailed below.

In phase three, covering the period 7-12 hpi, 80-90% of the total release of prelabelled, pelletable thymidine and methionine and 60-70% of the total release of X and NSI occurred. About a quarter of the total prelabelled [^{3}H]-thymidine in the cell culture was released from 7 -12 hpi. Free viral protein release was substantial (about 70% of total) and Coulter counted debris was at its highest value.

It seems likely therefore from this data that some, at least, of the release of free protein occurs due to a deterioration in the host cells. However, the level of release of uninfected cells showed that most of the damage is not virus induced though it could still result in the release of virus proteins. There are also other inconsistencies in implicating virus induced cellular permeability. Release of host components at the beginning of phase 3 (7-8½ hpi) was not accompanied by a corresponding level of free viral proteins whilst the later release (9-12 hpi) did result in both viral and host component release. The data on the transport of viral proteins from the nucleus also suggest that this process remains specific up to 11 hpi (see above). It is possible that the wash of cell monolayers prior to fractionation elutes off absorbed virus which thus appears in the medium in the transport experiments whilst it is still attached to the monolayer in the experiments analysing the tissue culture fluids.

Since influenza virus is released by budding through the plasma membrane, the release of labelled virions observed at this time could not be due to leakage of cytoplasm or sloughing off of whole cells. However, if virus was adsorbed to the plasma membrane as suggested above, then the changes which take place in the host cell at this stage

of infection might permit the release of some of this adsorbed virus due to the disintegration of the means of its adsorption.

4.G The Distribution of Proteins Incorporated into Virions

HA2 and NP are incorporated into virions at low levels after their synthesis, and this level rises to a peak at 6-7 hpi. In contrast, M is found in virions in roughly similar amounts from 3-7 hpi. The amount of labelled M and NP in virions again rises to a second maximum at 9-10 hpi, whilst the level of HA2 incorporation remains low throughout this period. The delay in appearance of NP and HA2 must reflect the processes to be accomplished with the newly synthesized polypeptide before its incorporation into the virion. NP must be assembled into the viral RNP which probably necessitates its transport into and out of the nucleus whilst HA2 must be cleaved from the precursor HA and glycosylated. The rapid appearance of M in virions suggests that there is immediate equilibration of the newly synthesized protein into the pool out of which virions are assembled and the constant rate of incorporation over the first four hours suggests that this pool is large.

These results are in complete agreement with Hay (1974) who measured cumulative totals of protein incorporated into virions. It has been suggested that the synthesis of matrix protein is limiting in virus multiplication and that the arrival of matrix protein at the plasma membrane may trigger the release of virus (Lazarowitz et al., 1971; Compans, 1973). However, our results, in agreement with Hay (1974), suggest that the synthesis of M is not rate limiting for there is a substantial pool of this protein both at 3 hpi (in our study) and at 4 hpi (in Hay's). However, there is a consensus between all these investigations that the incorporation of matrix protein into virions is the final process prior to budding. In extension of the study of Hay (1974), we have found further incorporation into virions of NP and M synthesized at $2\frac{1}{2}$ to 3 hpi at 9-11 hpi. The work of Meier-Ewert and Compans (1974) in which they found that maximal incorporation of NP and glycoproteins into virions came with proteins synthesized 2 h earlier than that of

matrix protein, is also consistent with this study. This indicates that at the time of maximal virion assembly, NP and glycoproteins are drawn from a pool of earlier synthesis than matrix protein.

4.H The Release of Free Protein

The only viral protein detected in the free protein fraction was NP, although traces of M could be observed at 9-11 hpi and NSI was visible at 3-4 hpi (Fig. 2.17). Whenever host protein X was released, it was found in the free protein fraction. About 65% of the NP released was in the soluble fraction and 10% of this was released in the first hour. It is probable that the unique distribution of NP amongst the viral proteins in this study may result not only from its association with the vRNA in virions but also its association with cRNA in the infected cell. Krug (1971, 1972) has found RNP's containing both v and cRNA in the cytoplasm and Pons (1971, 1972) has observed the addition of NP to incomplete cRNA's during transcription and concluded that all cRNA's were associated with NP. This cRNA-NP may thus be released from the cell when cRNA synthesis is declining. However, we have not analysed RNA's released from the cell.

4.I Release of Sub-viral Particles

These structures sedimented on a velocity gradient at a rate between that of virions and free protein but were never clearly resolved from the free protein fraction. Distribution of SVP's over the chase period was similar to the viral proteins in the soluble fraction, but it contained more M and NSI relative to NP and did not contain X at any time. We do not know whether this structure contains RNA or what its role might be. We have no evidence that we are analyzing the same structure throughout the period studied, although the fact that it differed in viral protein content from both the virion and soluble fractions argues for some uniformity. Further characterization of this entity by velocity gradient centrifugation and analysis of its RNA content are required before its relevance can be meaningfully assessed.

5. CONCLUSION

Implications of the Transport of NP, M and NSI into, and NP and M out of the Nucleus

This part speculates on the role of these proteins in virus replication and the reasons for their migration to and from the nucleus. The site of influenza RNA synthesis is controversial, but if the virion RNA were to be synthesized in the nucleus, then assembly of RNP's for incorporation into virions might take place at this site. Thus NP might move to the nucleus and, as vRNA was synthesized, become incorporated into an RNP structure which subsequently migrated through the cytoplasm to the site of virion assembly at the plasma membrane. The movement of radiolabelled protein in and out of the nucleus is consistent with this plan and the accumulation of antigen (probably RNP rather than NP) in the nucleus and its subsequent migration also support this idea. The results of this study suggest that, unlike M and NSI, a considerable portion of NP remains cytoplasmic. Given the knowledge that NP can associate with either v or cRNA, it is tempting to suggest that the cytoplasmic RNP contains predominantly cRNA and functions to protect cRNA and/or exert control on its translation. This might then indicate that a proportion of NP protein does not go through the nucleus but associates with cRNA in the cytoplasm to form cRNP antigen.

NSI is synthesized early in infection and accumulates in the nucleus, concentrated in the nucleolus (Dimmock, 1969; Krug and Etkind, 1973; Krug and Soeiro, 1975). It is found in the cytoplasm predominantly associated with polysomes (Compans, 1973; Klenk et al., 1974; Hay, 1974; Pons, 1972) although it does not behave as a ribosomal structural protein (Klug and Etkind, 1973). However, this almost exclusive association with ribosomal processes suggests a role in viral protein synthesis or possibly transcription, especially if any of the viral mRNA's make use of rRNA synthesis machinery as suggested by the inhibitor studes of Minor and Dimmock (1977). Its early synthesis,

the decrease in this synthesis by 4 hpi and its accumulation in the nucleus only early on tentatively suggest that it may be required for an early step in virus mulitplication such as the early-late protein switch (Skehel, 1973).

/Here I suggest a controversial scheme which explains the movements of matrix protein. M is synthesized in the cytoplasm and accumulates rapidly in the nucleus where a large pool develops. A small amount of M is not transported, and accumulates on the plasma membrane due to an affinity with this structure. The assembly of RNP's containing NP and the P proteins is completed by the association of M and the resulting core is rapidly transported through the cytoplasm and assembled into virions whose membrane is specified by the glycoproteins alone. Contrary to the accepted dogma, in this scheme, it is the cytoplasmic matrix protein which is redundant and the nuclear protein which is utilized in virions. How does this scheme accord with the evidence ?

The accumulation of M in the nucleus is rapid as shown by this study and by Hay and Skehel (1975) who found that, after a 2 min pulse of M, transport to the nucleus was virtually complete within 15 min. That there is a large pool of M in the infected cell is suggested by these studies and others (Compans, 1973; Klenk et al., 1974· Hay, 1974). Hay found that M was present in the nuclei at high levels at 10 hpi, though the site of the pool is not investigated in other studies. The present study has demonstrated the movement of M from the nucleus at a similar rate to that of NP. M does not accumulate in the cytoplasm following this transport, but appears in the medium in virions. This is consistent with a rapid and co-ordinated transport of NP and M through the cytoplasm.

The scheme would explain the failure of all studies to detect M in association with RNP structures containing NP and P proteins in the cytoplasm (Compans, 1973; Klenk et al., 1974; Hay and Skehel, 1975) since these are probably cRNA containing and involved in translation.

Some RNP's may appear in the cytoplasm containing vRNA either by abortive assembly and transport or in connection with cRNA synthesis. The apparent movement of NP antigen (this thesis; Breitenfeld and Schafer, 1957; Maeno and Kilbourne, 1970) might reflect loss of M-RNP from the nucleus, but the rise in cytoplasmic levels could be due to RNP containing cRNA. It is also possible that some of the cytoplasmic fluorescence is in fact at the plasma membrane representing budding or reabsorbed virus. Cores containing matrix protein have been found after treatment of virions with detergents (Reginster and Nermut, 1976).

The accumulation of M in association with rough ER might be due to its synthesis and a limited availability of transport processes to the nucleus whilst its accumulation at the plasma membrane is abortive as regards incorporation into virions. This is supported by the failure to chase M out of smooth membrane and plasma membrane fractions (Compans, 1973; Klenk et al., 1974; Hay, 1974). The concept advanced that the arrival of M triggers the budding of the virus particles is consistent with this scheme (Lazarowitz et al., 1971). An apparent discrepancy with this scheme arises since in this study and in a previous work (Hay, 1974) M appeared to be incorporated into virions from its time of synthesis. But in our study a pulse of 30 min allowed ample time for nuclear accumulation, equilibration with the pool of matrix protein and subsequent core transport, whilst Hay used a pulse of 15 min and analysed the virions produced after 30 min, again enough time for the rate of M utilization to attain its steady state value. Though Hay found the incorporation of matrix protein consistent with a putative role in the formation of the virion envelope, he did not exclude some other role for this protein on account of its substantial accumulation in the nucleus. Evidence for the presence of RNP structures containing M is presented in Section 3.

This hypothesis is open to test by manipulating the conditions so that the hypothetical M containing RNP (M-RNP) might accumulate. Infected cells could be treated with anti-neuraminidase which will result in patching and capping of the influenza NA as it is expressed. This will inhibit virion budding without affecting intracellular processes and thus an accumulation of M-RNP might be observed in the cytoplasm. A similar rationale would apply to the use of a ts mutant defective in HA and NA synthesis. Alternatively one could use an L-cell system which allows the infection cycle of influenza to proceed but the NP antigen is not observed to migrate from the nucleus. In this situation, M-containing RNP's might build up in the nucleus. The concentration of M-RNP formed by these procedures could then be analysed by gradient centrifugation.

Section 3 : The Distribution of Viral Proteins within the Nucleus and Cytoplasm: Location of Viral Proteins in Substructures

1. Introduction

The use of Metrizamide as a gradient centrifugation medium has considerable advantages over conventional media. It is completely non-ionic, an advantage over caesium chloride, yet it is considerably less viscous than sucrose. For any given concentration it is denser than sucrose but, like sucrose, it exhibits a near linear relationship between concentration and osmolarity. Its refractive index provides a ready measure of its concentration and hence its density. Gradients can be formed simply by sedimentation, unlike sucrose, but these form more slowly than those of caesium chloride (Rickwood, 1976).

The density of components in Metrizamide is determined mainly by their degree of hydration, the greater the hydration the lower the density of the component (Birnie et al., 1973). The use of Metrizamide gradients to resolve RNP's has been described (Rickwood and Birnie, 1975) and this method enables RNP's to be separated according to the ratio of RNA to protein. Chromatin is also separated into discrete peaks (Rickwood et al., 1973).

Previous analyses of influenza RNP's in infected cells had generally concentrated on the RNA in such structures and little was known of the protein composition of such RNP's. In most studies it was found necessary to fix the RNP's with glutaraldehyde (Krug, 1971) or formaldehyde (Assadullaeff et al., 1975) to prevent disruption of components with all its attendant artefacts. Furthermore, the isolation of RNP's by several step methods made it likely that components might be lost or rearranged and that fragile structures might be irreversibly disrupted. Some extractions involve treatment with DNAse which prevents the investigation of any association with cell chromatin.

It seemed that an analysis of total cell compartments with the minimum of preparative steps using a method which might preserve the association of viral components in viral structures and in association with host cell components was warranted. Metrizamide

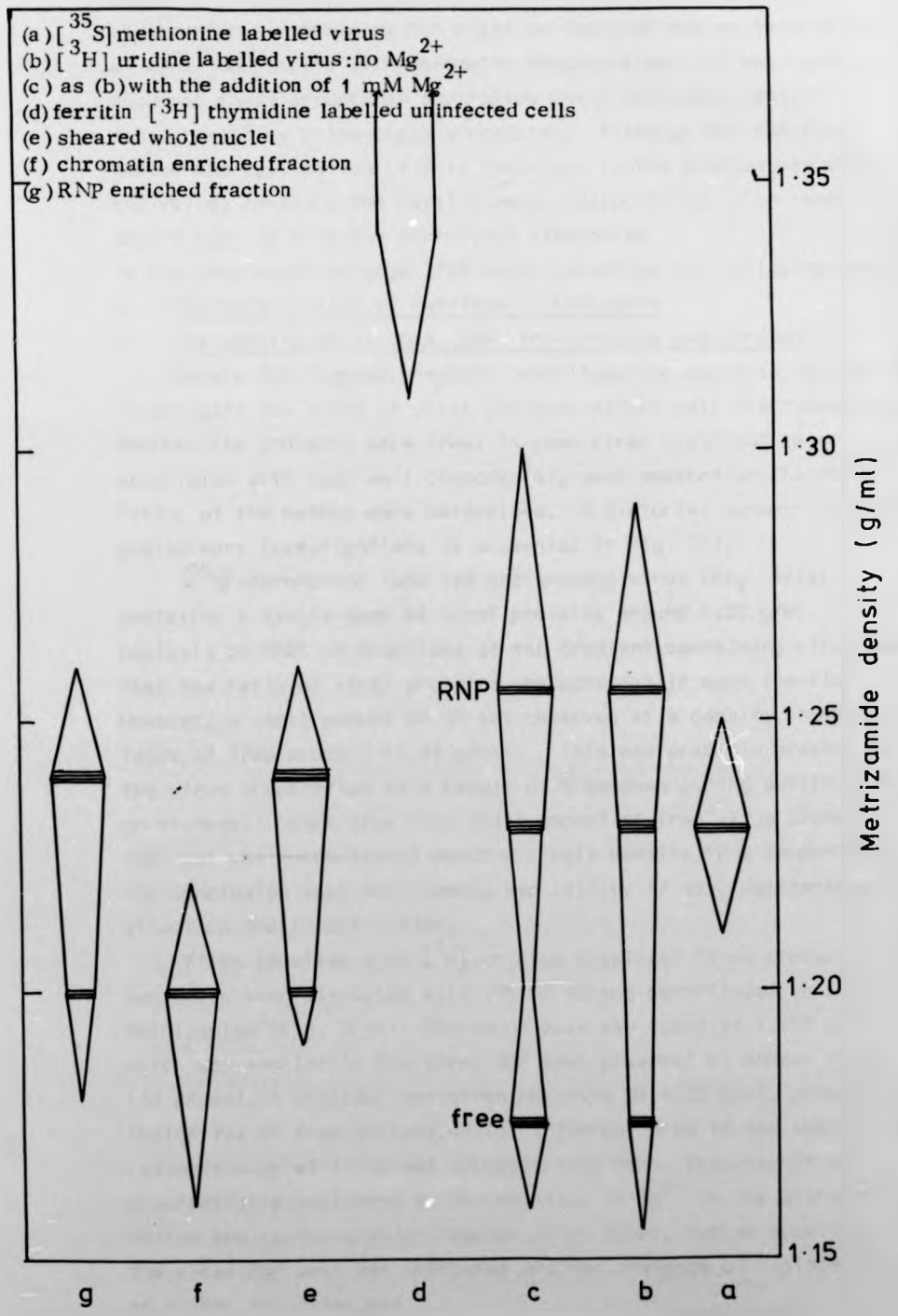


Fig. 3/1 Schematic representation of the density of some viral and cell structures in Metrizamide gradients.

appeared to offer a unique opportunity to study the state of viral proteins within the infected cell. In the nucleus, putative virion substructures containing RNA might be isolated and an association of viral components with chromatin demonstrated. It was hoped to document these structures and follow their synthesis, assembly and movement by pulse-chase procedures. Although the rationale behind the application of this technique to the problem was sound and valid, however, the results were disappointing. The reader should bear in mind the strictures elaborated in the conclusion on page 3/32 when evaluating the following results.

2. Characterization of Metrizamide Gradients

A. The density of virions, RNP, free protein and chromatin

Before Metrizamide gradient centrifugation could be applied to investigate the state of viral proteins within cell fractions (i.e. whether the proteins were free, in some viral substructure or associated with host cell components), some separation characteristics of the method were determined. A pictorial summary of these preliminary investigations is presented in Fig. 3/1.

[^{35}S]-methionine labelled undisrupted virus (Fig. 3/1a) exhibited a single peak of viral proteins around 1.22 g/ml. Analysis by PAGE of fractions of the gradient containing virus showed that the ratio of viral proteins was constant in each fraction. However, a small amount of NP was observed at a density characteristic of free protein (1.35 g/ml). This was probably present in the virus preparation as a result of breakdown during purification or storage. Apart from this small amount of free virus protein, the rest was concentrated about a single density thus encouraging the conclusion that Metrizamide had little, if any, deleterious effect on the intact virion.

Virus labelled with [^{3}H]-uridine displayed three characteristic densities when disrupted with 10% NP 40 and centrifuged in 37% Metrizamide (Fig. 3/1c). The major peak was found at 1.255 g/ml which was similar to the viral RNP peak observed by Hudson et al. (in press). A shoulder was often observed at 1.22 g/ml, presumably indicative of free virions,whilst a further step in the profile of radioactivity at 1.175 was probably free RNA. This result was substantially unaltered by the omission of Mg^{2+} in the disruption medium and in the gradient buffer (Fig. 3/1b), but on occasions the viral RNP peak was sharpened and the presence of radiocactivity at higher densities was

reduced. However, since all the fractionation studies and all virus and cell buffers contained magnesium,which enhances transcriptase activity and hence may stabilize RNP's, it was decided to incorporate it into the Metrizamide gradients. It was also hoped to clear polysomes from the critical densities (1.30-1.20 g/ml) of the gradient since these might contain nascent viral polypeptides in the shorter chase samples. These had been shown to be shifted from 1.24 to 1.35 g/ml by the addition of $MgCl_2$ to the gradient medium (Buckingham and Gross, 1975).

Ferritin, taken as illustrative of free protein, was found at densities in excess of 1.31 (Fig. 3/1d) whilst cytochrome c and haemaglobin also exhibited considerable complexing with Metrizamide and were found at high densities or precipitated (density > 1.38 g/ml). However the observation of material at these high densities (Birnie et al., 1973; Rickwood et al., 1974; Rickwood and Birnie, 1975), predominantly free protein complexed with Metrizamide, renders questionable the assumption that protein at these densities is unambiguously free in its native state. However, repeatable differences were found between material in this region and at lower densities in the following studies.

The behaviour of chromatin is summarized in Fig. 3/1e, f, g. Whole nuclei, sheared by repeated passage through a 26 G syringe needle, exhibited a major peak at 1.24 g/ml with a shoulder at 1.20 g/ml in agreement with the results of other workers (Birnie et al., 1973; Rickwood et al., 1974b). If the nuclei were further fractionated by multiple isotonic salt washes to extract an 'RNP enriched' fraction and leave chromatin, the 1.20 g/ml peak alone was found in the chromatin fraction whilst, in the RNP fraction, the higher density component was predominant. It appears that the more vigorous the disruptive treatment the greater is the loss of the denser component (Rickwood et al., 1974b), thus Hudson et al. (in press) found chromatin only at 1.20 g/ml.

The banding of membranes in Metrizamide is poorly documented but it has been reported (Aas, 1973) that microsomal membranes band

(a) Disrupted in 10% NP 40 for 30 min at room temperature
(b) Disrupted in 1% NP 40 for 5 min at 4^{o}
(c) Disrupted in 1% NP 40 and 0.5% DOC for 4 min at 37^{o}

○ : NP; ● : M ; □ : HA2; ⊢—⊣ position in which P's were discernible.

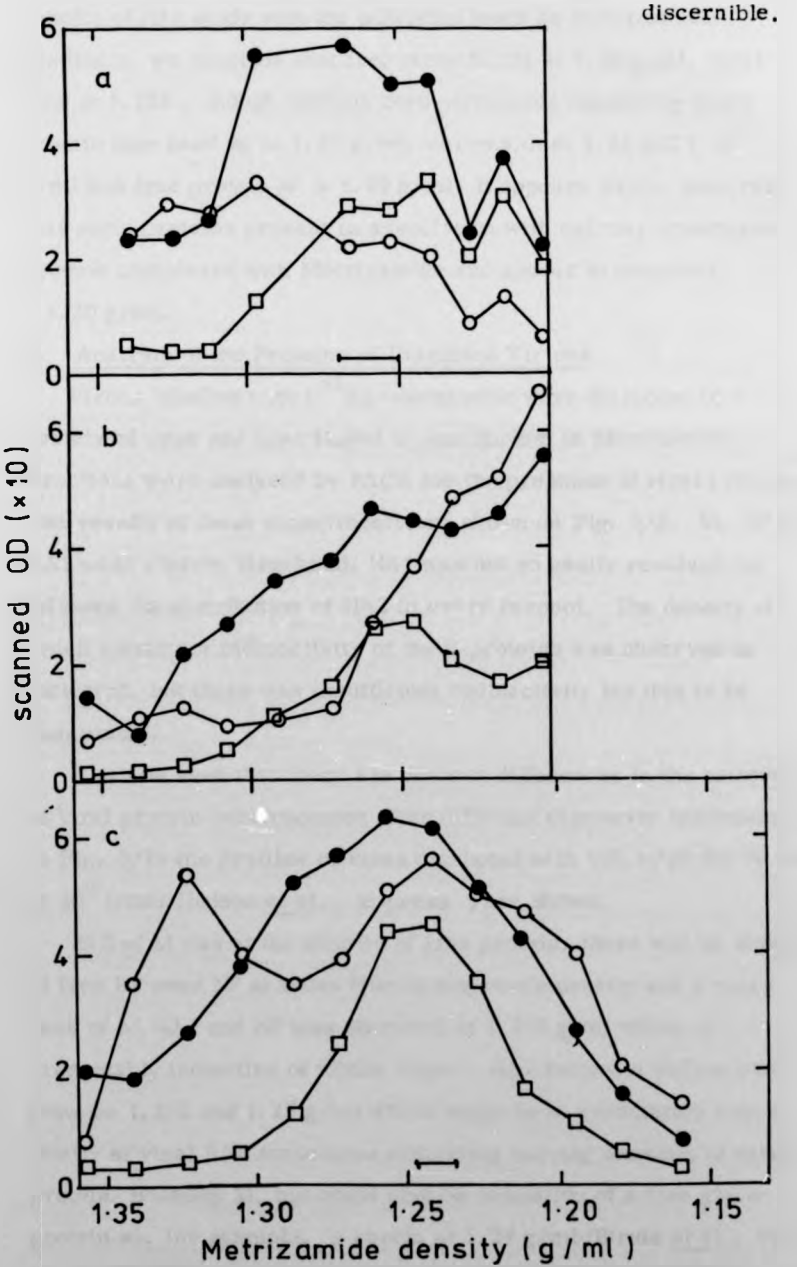


Fig. 3/2 Profiles obtained from Metrizamide gradient centrifugation of disrupted [^{35}S] methionine labelled virus.

heterogeneously between 1.14 and 1.26 g/ml. Combining the results of this study with the published work on Metrizamide gradients, we conclude that free virus bands at 1.22 g/ml, viral RNP at 1.255 , though various core structures containing more protein may band up to 1.27 g/ml, chromatin at 1.24 and 1.20 g/ml and free protein at > 1.29 g/ml. It appears likely, however, that some proteins present in association with cellular structures become complexed with Metrizamide and appear at densities > 1.30 g/ml.

B. Analysis of the Proteins of Disrupted Virions

Virons labelled with [^{35}S] -methionine were disrupted in a variety of ways and centrifuged to equilibrium in Metrizamide. Fractions were analyzed by PAGE for the presence of viral proteins. The results of these experiments are shown on Fig. 3/2. M, NP and HA2 were clearly visualized; HA1 was not so easily resolved but followed the distribution of HA2 in every respect. The density at which maximum radioactivity of the P proteins was observed is indicated, but there was insufficient radioactivity for this to be quantitated.

It can be seen that there are distinct differences in the patterns of viral protein substructures after different disruptive treatments. In Fig. 3/2a the profiles of virus disrupted with 10% NP40 for 30 min at 20^{o} (after Hudson et al., In press) are shown.

25% of M was at the density of free protein, there was an excess of free NP over NP at virus ribonucleoprotein density and a small peak of M, HA2 and NP was observed at 1.215 g/ml which is presumably indicative of whole virus. HA2 formed a diffuse peak between 1.235 and 1.27 g/ml which might be an association with a family of viral RNP structures containing varying amounts of extra protein, possibly M, but could also be indicative of a free glycoprotein as, for example, α casein at 1.24 g/ml (Birnie et al., 1973). Thus 10% NP40 treatment produces substantial amounts of free protein but also gives rise to a range of structures at the densities of RNP's.

In contrast to the vigorous treatment above, a procedure involving exposure to 1% NP40 for very limited periods of time at 4^{o} (adapted from Almeida and Brand, 1975) produced much less free M and very little free NP (Fig. 3/2b). There was a slight peak of M at viral RNP density but little NP. By far the greatest amount of NP was at free virus density where there was also considerable matrix protein. The glycoproteins appeared much as in the above treatment whilst P proteins were concentrated at a density of 1.22-1.23 g/ml corresponding to that of free virus. Thus this treatment produces some RNP density material,but most viral protein is found in the region of free virus indicating that this limited treatment does not disrupt a considerable proportion of the virions. We also used a cocktail of NP40 and sodium deoxycholate for 4 min at 37^{o} (Pons, 1971). This produced the profiles shown on Fig. 3/2c. There was a large peak of M and NP at viral RNP density where the P proteins were also detectable. Curiously there was also a peak of free NP at a high density of 1.325 g/ml which was not observed with the other disrupted virus samples. This may be indicative of an NP-Metrizamide complex (Rickwood et al., 1974a).

A shoulder of M protein appears at 1.29 g/ml and the profiles of both M and NP suggest that free virus is also still present at 1.22-1.23 g/ml. HA2 appeared in a peak at 1.25 g/ml. The method of Pons (1971) gave a good yield of viral proteins at typical viral RNP density and is the preferred method of the described sets of disruption conditions.

In summary, the disruption conditions have a profound effect on the distribution of viral proteins found as free proteins in vRNP structures and in whole virions. The glycoproteins appear always to be solubilized. Whilst 10% NP40 treatment produces much free protein and a high M:NP ratio in vRNP's disruption by the cocktail of detergents yields more vRNP but with less M relative to NP. All the methods suggest that structures with a density range between free protein at > 1.29 and the vRNP at 1.255 g/ml are always present. Some whole virus remains and all methods yield a significant proportion of free internal viral proteins.

3. The State of Newly Synthesized Viral Proteins in the Nucleus

After its initial characterization, Metrizamide gradient centrifugation was employed to probe the state of viral proteins present in the nucleus. It was hoped to determine if there were one or more RNA-containing substructures at densities between 1.22 and 1.27 g/ml which might be involved in the putative nuclear production and packaging of influenza virion RNA, to analyze the viral protein complement of these structures and to investigate their fate. In all these studies it was hoped to isolate the putative core structures (see Section 2.4 & 5) before their transport into virions.

3.A. A Comparison of the State of Viral Proteins after Synthesis at 2 and 5 hpi.

In one group of experiments three sets of conditions were investigated: (1) the distribution of viral proteins shortly after their synthesis at 2 hpi, (2) that of viral proteins shortly after their synthesis at 5 hpi and (3) the distribution of proteins at 5 hpi which were synthesized at 2 h after infection. Two events are initiated between these times. One is the initial rapid rise in intracellular infectivity (3 $\log_{10}$ between 2 and 4 hpi; see Fig. 2/21). The other is the assymetric distribution of NP observed by immunofluorescence between cytoplasm and nucleus. NP is observed initially at 2 hpi in the nucleus and subsequently equalizes between the nucleus and cytoplasm ($3\frac{1}{2}$-5 hpi)(see Section 2.2C). In comparing the state of viral proteins at the beginning and at the end of the period when the large increase in intracellular infectivity was occurring, it was hoped to find the core structure envisaged in Section 2 (and any other SVP which might be present at the early timepoint but not the later one). Quantitative differences in the proportions of proteins in different structures might be highlighted with an indication of their relevance to virus multiplication.

Pulsed at 2 hpi for 10 min and chased for 20 min: Nuclei
(a) ○ NP (b) ■ NSI

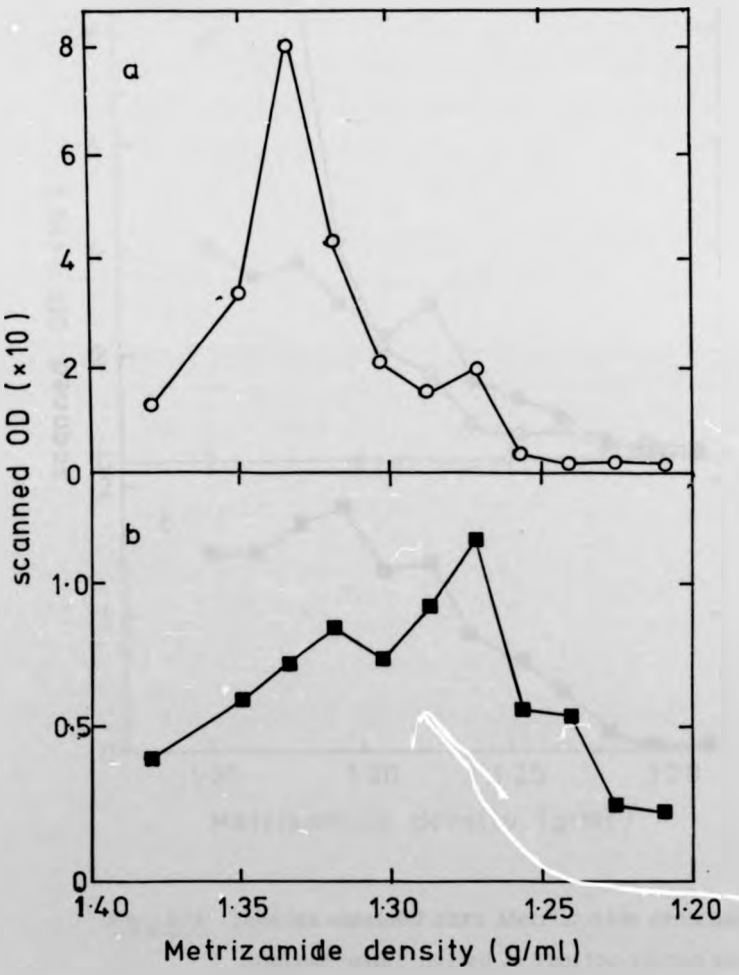


Fig. 3/3 Profiles obtained after Metrizamide centrifugation of infected cells.

Cells were infected for 15 min at room temperature and incubated. After pulse and chase, the cells were fractionated by NML and the nuclei and cytoplasmic fractions layered on to Metrizamide. After centrifugation, the gradients were fractionated and the proteins precipitated from each fraction. These were prepared and analyzed on 10% phosphate PAGE.

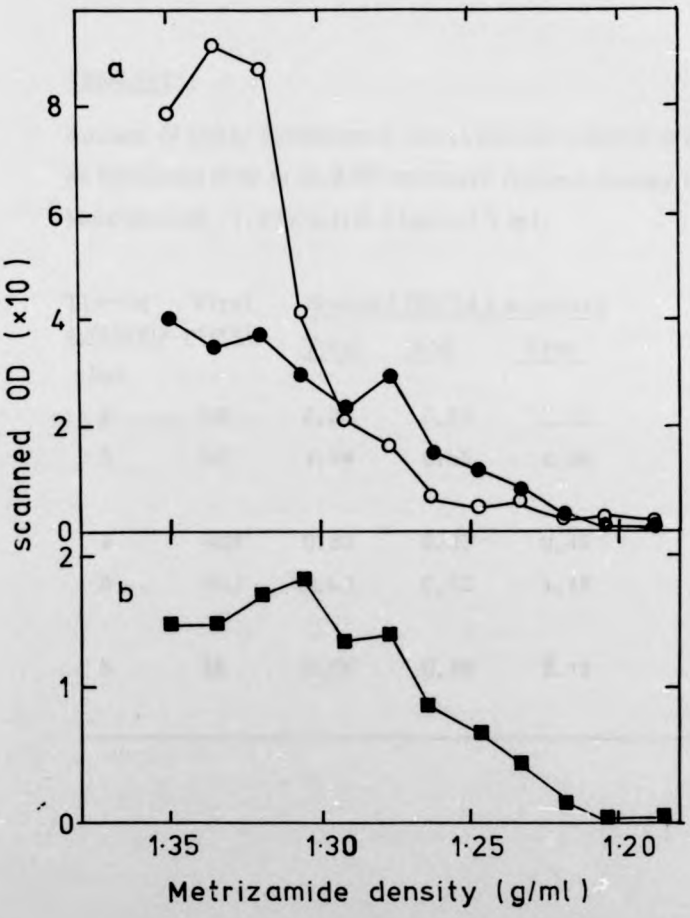


Fig. 3/4 Profiles obtained after Metrizamide centrifugation of infected cells: pulsed at 5 hpi for 10 min and chased for 20 min: Nuclei.

Method: see Fig. 3/3.

(a) ○ NP
● M
(b) ■ NSI

Table 3/1

Amount of newly synthesized viral proteins present in the nuclei as free protein or in an RNP structure (typical density in Metrizamide, 1.27 g/ml) at 2 hpi and 5 hpi.

Time of synthesis	Viral protein	Scanned OD/24 h exposure			% of total in RNP
		Total	RNP	Free	
hpi					
2	NP	3.30	0.58	2.72	18
5	NP	4.94	0.55	4.39	11
2	NSI	0.82	0.37	0.45	45
5	NSI	2.00	0.52	1.48	26
5	M	3.01	0.89	2.12	30

The Distribution of Viral Proteins at 2 hpi compared with that at 5 hpi

Cells were infected with FP/Rostock (moi = 20) and incubated for 2 or 5 h. The cells were pulsed and chased for 20 min, nuclear monolayers were prepared and the nuclei were removed by scraping, sheared and layered on to 37% Metrizamide. This was centrifuged to equilibrium at 4^{o} and fractionated. The refractive index of every third fraction was measured and the fractions were then precipitated with ethanol and resuspended in sample buffer . The samples were analyzed on PAGE. After fluorography the autoradiographs were scanned and peak heights determined. These are plotted against Metrizamide density in Fig. 3/3 and 4.

At both timepoints NP and NS1 were found predominantly at a density $\geq$ 1.30 g/ml but there was a significant accumulation of NP at a density of 1.27 g/ml at 2 hpi. This is indicative of an RNP structure with a higher protein to RNA ratio than the major RNP extractable from virions (density, 1.255 g/ml). At the later timepoint there appeared to be less NP in this structure. NS1 also appeared as a peak at this density (Fig. 3/3b) and after the 5 hpi pulse, when matrix protein was detectable, it was also concentrated at 1.27 g/ml (Fig. 3/4a).

In order to obtain a crude quantitation of this data, the OD between 1.30 and 1.35 g/ml for each protein was summed and taken to be a measure of its free and Metrizamide complexed form whilst the OD at 1.26-1.29 g/ml was totalled to provide a value for RNP structures (Table 3/1). There was considerably more of both newly synthesized NP and NS1 in the nucleus at 5 hpi than at 2 hpi, but most of this extra accumulation appeared as free protein. Thus, although the percentage of total protein present as RNP fell from 18 to 11% for NP and 45 to 26% for NS1, the actual amount of NP in RNP remained the same whilst NS1 increased from 0.37 to 0.52 OD units. Though the two proteins appeared similar inthe above respect, the distribution between RNP and free protein was markedly different. At 2 hpi, 45% of NS1 was present in an RNP whilst this structure contained only 18% of NP. M was also found at the RNP density (1.27 g/ml) (Table 3/1 and Fig. 3/4a) but again most (70%) was at the density of free protein. It must be stressed that this quantitation is on a very crude basis and cannot be used to establish accurate levels.

Table 3/2

Amounts of newly synthesized viral proteins present as free protein or in an RNP structure from an RNP enriched fraction.

Time of synthesis hpi	Viral protein	Scanned OD/24 h exposure				% of total in RNP	
		Total	RNP at 1.27 g/ml	RNP at 1.23 g/ml	Free protein	at 1.27 g/ml	at 1.23 g/ml
2	NP	2.06	0.23	0.05	1.78	11	2
5	NP	4.67	1.22	0.60	2.85	26	13
2	NSI	0.89	0.36	0.07	0.46	40	8
5	NSI	1.24	0.49	0.23	0.52	40	19
5	M	1.34	0.46	0.28	0.60	34	21

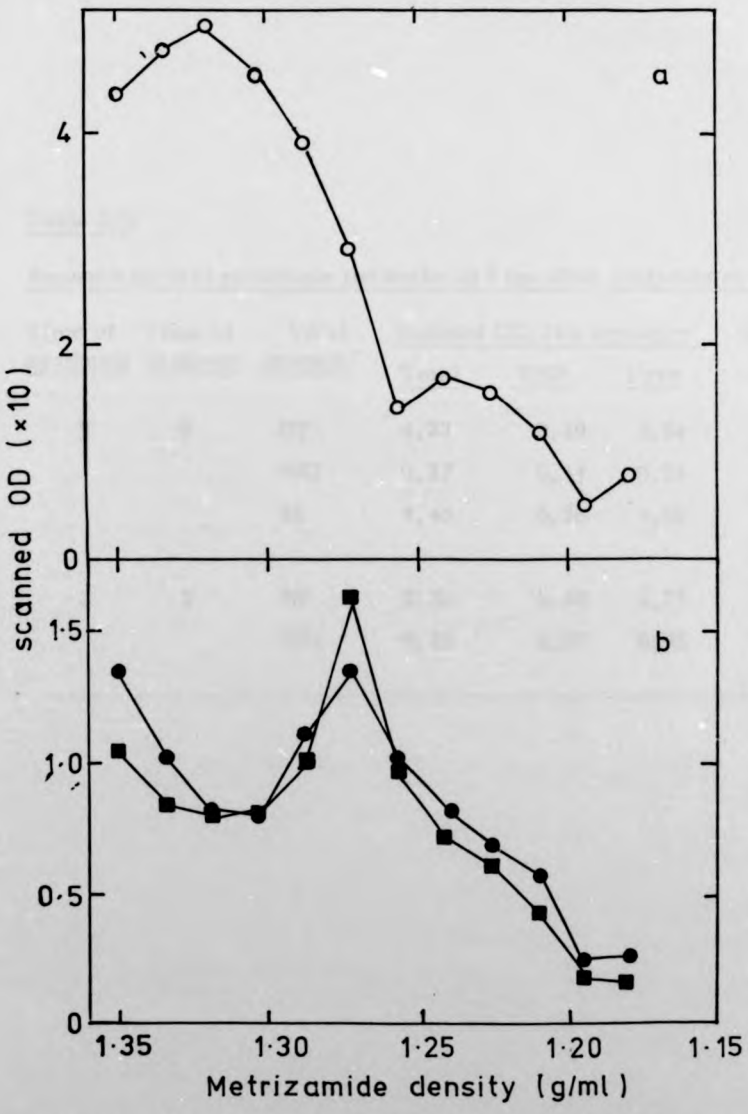


Fig. 3/5 Profiles obtained after Metrizamide centrifugation of infected cells pulsed at 5 hpi for 10 min and chased for 20 min: RNP enriched fraction.

(a) ○ NP
(b) ● M
■ NSI

Table 3/3

Amounts of viral proteins in the nuclei at 5 hpi after synthesis at 2 hpi

Time of synthesis	Time of analysis	Viral protein	Scanned OD/24 h exposure			% of total in RNP
			Total	RNP	Free	
2	5	NP	4.23	0.39	3.84	9
		NSI	0.37	0.11	0.26	30
		M	1.46	0.36	1.10	25
2	2	NP	3.30	0.58	2.72	18
		NSI	0.82	0.37	0.45	45

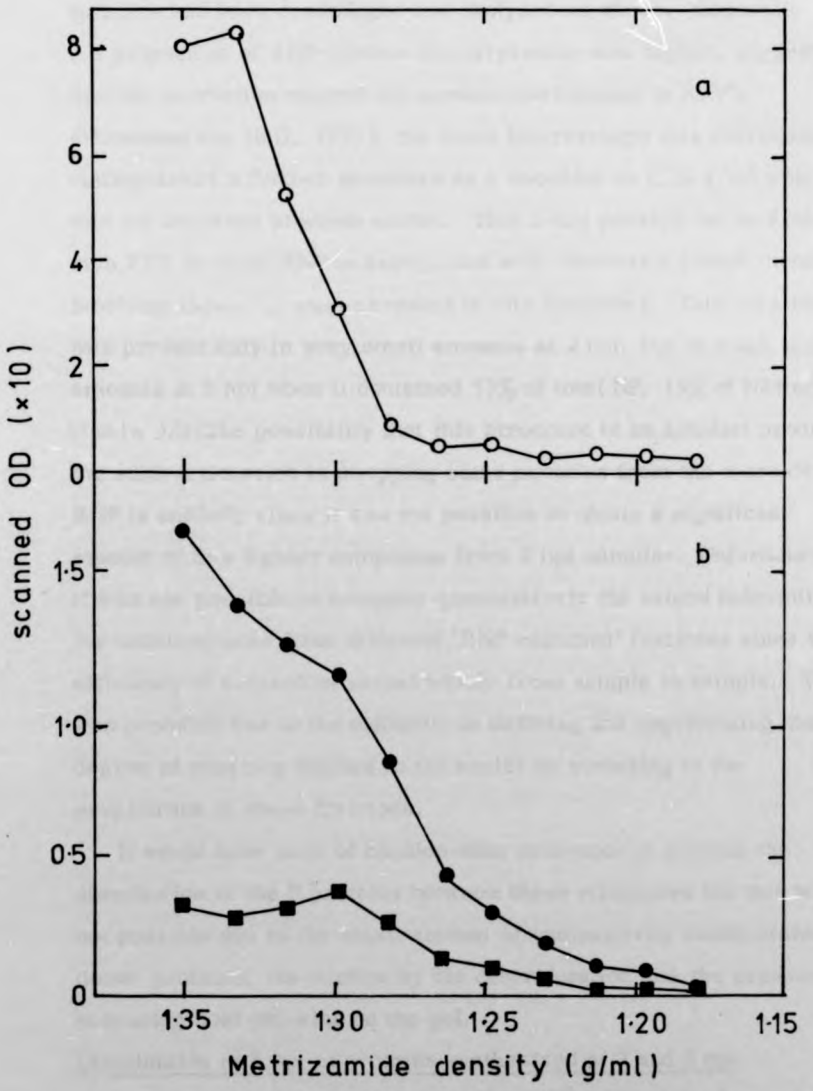


Fig. 3/6 Profiles obtained after Metrizamide centrifugation of infectedcells. Pulsed at 2 hpi for 10 min and chased to $5\frac{1}{2}$ hpi: Nuclei (a) ○ NP (b) ● M ■ NS1

Further fractionation of the nuclei by isotonic salt wash to extract an 'RNP enriched' fraction gave the results presented in Table 3/2 (for an example of profiles, see Fig. 3/5) when the samples had been centrifuged and analyzed as above. Generally the proportion of RNP-protein to total protein was higher, suggesting that the extraction method did produce enrichment in RNP's (Monachan and Hall, 1975), but more interestingly this extraction distinguished a further structure as a shoulder at 1.23 g/ml which was not resolved in whole nuclei. This could possibly be an RNA-rich RNP or viral RNP in association with chromatin (which thymidine labelling shows is always present in this fraction). This structure was present only in very small amounts at 2 hpi, but in much larger amounts at 5 hpi when it contained 13% of total NP, 19% of NS1 and 21% of M (Table 3/2) The possibility that this structure is an artefact produced by the subfractionation in stripping some proteins from the more dense RNP is unlikely since it was not possible to obtain a significant amount of this lighter component from 2 hpi samples. Unfortunately it was not possible to compare quantitatively the values determined for substructures from different 'RNP enriched' fractions since the efficiency of extraction varied widely from sample to sample. This was probably due to the difficulty in defining and regularizing the degree of shearing applied to the nuclei by vortexing in the preparation of these fractions.

It would have been of considerable relevance to analyse the distribution of the P proteins between these structures but this was not possible due to the small amount of radioactivity incorporated in these proteins, its dilution by the centrifugation and the presence of obscuring host proteins on the gel.

Distribution at 5 hpi of proteins synthesized at 2 and 5 hpi

The profile of the long chase (Fig. 3/6 and quantitation table 3/3) shows that little NP (9%) is banding at 1.27 g/ml, only 45% of the NS1 synthesized at 2 hpi is left in the nucleus and even less at that density (30% compared with 45% at 2 hpi) whilst matrix

protein appears predominantly (75%) free or Metrizamide complexed. Thus it seems that the viral RNP present at 1.27 g/ml at 2 hpi has disappeared after chasing to 5 hpi though there is still a large amount of protein present at density >1.30 g/ml. There was no evidence for any remaining 1.23 g/ml structure as an RNP extraction yielded only NP at high density. It is curious that matrix protein should be observed since it is only just being synthesized at 2 hpi and must presumably be due to its synthesis, subsequent to the pulse, from the pool of radioactive methionine. This has been observed before (see Fig. 2/11). Alternatively this might represent M protein in the cytoplasm which has slowly accumulated in the nucleus, though this is unlikely due to the timing.

3. B. Comparison of the State of Viral Proteins between $3\frac{1}{2}$ and 6 hpi

From the foregoing part, it appeared that differences could be observed in vRNP substructures between times which lay before and after the rapid rise in infectivity in the virus multiplication cycle. In order to follow the assembly and movement of these substructures, a single pulse time was selected and a series of cultures were chased for varying lengths of time. The cultures were then fractionated into nucleus and cytoplasm and processed on Metrizamide gradients as before. So that the substructures could be followed outside the nucleus if this occurred, both nuclear and cytoplasmic fractions were analyzed on the gradients, although analysis of the latter is complicated by the variety of structures present, particularly membranes and polysomes.

At $3\frac{1}{2}$ hpi the synthesis of matrix protein is in progress and virus synthesis is exponential. Since the distribution of matrix protein was of interest, this timepoint was chosen for further study with chase times from 5 min to $2\frac{1}{2}$ h.

A number of problems were encountered in these experiments which resulted in less clearcut observations than in 3.A. There was a very large excess of protein synthesized at these timepoints which banded at a density greater than 1.30 g/ml and this rendered difficult the analysis of the RNP density range in which comparatively

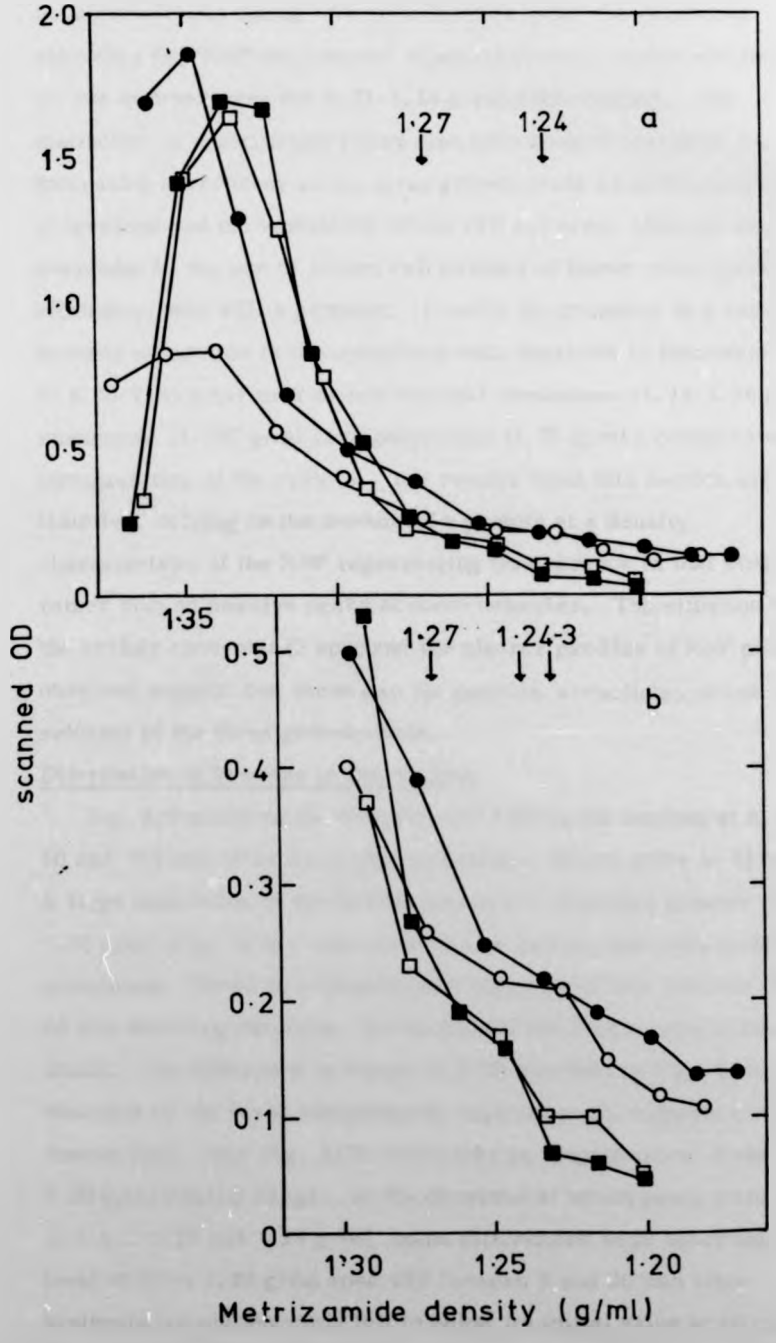


Fig. 3/7 Profiles obtained after Metrizamide centrifugation of infected cells. Pulsed at $3\frac{1}{2}$ hpi for 10 min and chased for up to $2\frac{1}{2}$ h: NP (a) in nucleus after chase: ○ 5 min
● 20 min
□ 60 min
■ 150 min
(b) Enlargment of scanned OD scale over 1.30-1.20 g/ml density range.

little protein was found. As mentioned earlier, the extraction efficiency for 'RNP enrichment' appeared to vary, which precluded its use in monitoring the 1.23-1.24 g/ml RNP complex. The distinction of substructures may also have been obscured by the increasing asynchrony in the virus growth cycle as multiplication progressed and the variability of the cell cultures, although partly overcome by the use of frozen cell batches of known virus uptake efficiency, was still a problem. Finally the presence of a variety of other structures in the cytoplasm with densities in Metrizamide of 1.20-1.35 g/ml such as microsomal membranes (1.14-1.26 g/ml), ribosomes (1.305 g/ml) and polysomes (1.35 g/ml) complicates the interpretation of the results. The results from this section are thus tentative, relying on the amount of a protein at a density characteristic of the RNP representing its presence in that structure rather than on positive peaks at these densities. The situation at the earlier timepoint (2 hpi) and the clearer profiles of RNP peaks observed suggest that these may be genuine structures which are relevant to the virus growth cycle.

Distribution of Proteins in the Nucleus

Fig. 3/7 presents the distribution of NP in the nucleus at 5, 20, 60 and 150 min after its synthesis during a 10 min pulse at $3\frac{1}{2}$ hpi. A large proportion of the protein appears at densities greater than 1.30 g/ml (Fig. 3/7a) indicative of free protein and protein-Metrizamide complexes. There is a considerable increase in this fraction from 5 to 20 min following the pulse, but thereafter the levels remain fairly stable. The difference in values at RNP densities in Fig. 3/7a is obscured by the scale compression required to incorporate the high density peak, thus Fig. 3/7b illustrates an amplification of the 1.20-1.30 g/ml density range. At the densities at which peaks were found in 3.A., 1.27 and 1.24 g/ml, some differences were observed. The level of NP at 1.27 g/ml rose 40% between 5 and 20 min after synthesis but subsequently fell to below its initial value at 60 and 150 min. A further difference was observed at 1.23-1.24 g/ml when the levels of NP remained at a stable value until after 20 min whereupon it dropped to 50% of this value at 60 min and 30% at 150 min.

Profiles obtained after Metrizamide centrifugation of infected cells.

Fig. 3/8 Pulsed at 3½ hpi for 10 min and chased for up to 2½ h: M in nucleus.

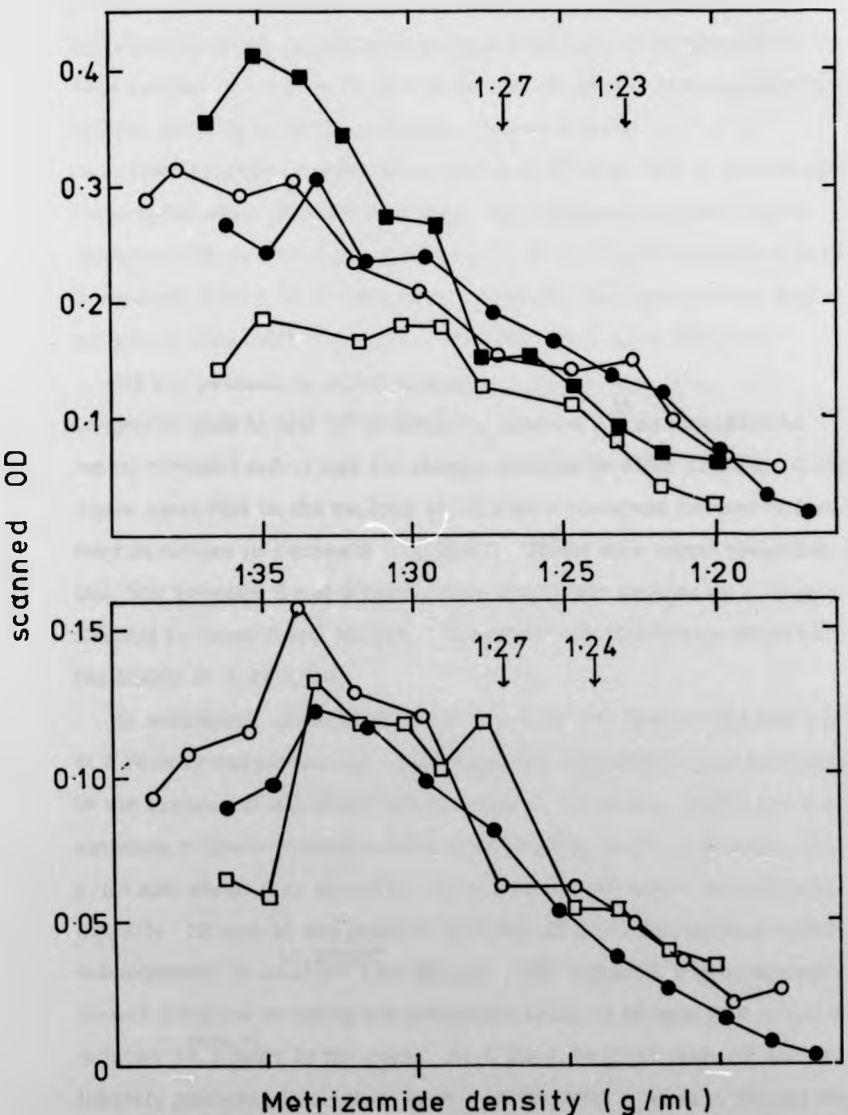


Fig. 3/9 Pulsed at 3½ hpi for 10 min and chased for up to 60 min: NSI in nucleus.

- ○ 5 min chase
- ● 20 min chase
- □ 60 min chase
- ■ 150 min chase

Matrix protein profiles (Fig. 3/8) show only small changes over the chase period. There was generally a smaller proportion of M present , free or complexed with Metrizamide,compared with NP but most of the M was present at high densities (1.30 upwards). This amount decreased from 5 to 60 min of chase, but reached its highest level $2\frac{1}{2}$ h after synthesis. The amount at 1.27 g/ml increased slightly in level between 5 and 20 min, fell at 60 and had risen again after 150 min of chase. It is doubtful whether these changes (20% or less) are significant. At 1.23 g/ml the level of M decreased from 5 to 60 min after synthesis, falling by some 50% of its initial value and remained at this low level up to 150 min.

NS 1 is present in small amounts in the nucleus (Fig. 3/9) compared with M and NP (at least by relative [^{35}S] -methionine incorporation) and it was not always detectable after 150 min of chase. Again most NS1 in the nucleus at all times occurred spread diffusely over densities in excessof 1.30 g/ml. There was some reduction in this NS1 between 5 and 20 min chase whilst the amount at 1.27 g/ml doubled between 5 and 60 min. Very little variation was observed in the levels at 1.24 g/ml.

In summary, most of the viral proteins NP, M and NS1 are present at a density characteristic of free and Metrizamide complexed protein in the nucleus at all of the times studied. However, there are small amounts of these proteins which comigrate at 1.27 g/ml and at 1.23-4 g/ml and which may therefore be in RNP complexes. At 1.27 g/ml initially NP and M are present at 5 and 20 min after protein synthesis subsequently to decline in amount by 60 min. NS1 appears at that density with slower kinetics reaching its maximum value at 60 min and similarly is reduced in amount later in the cycle. At 1.23-1.24 g/ml both NP and M are initially present, but their levels also drop after a 60 min chase.

Distribution of Proteins in the Cytoplasm

In the cytoplasm NP, matrix, NS1 and HA2 were present in sufficient concentration to be analyzed. The profile of NP (Fig. 3/10) showed a remarkably similar profile for 5, 20 and 150 min chases whilst that at 60 min showed more free NP and correspondingly less

Profiles obtained after Metrizamide centrifugation of infected cells.
Fig. 3/10 Pulsed at $3\frac{1}{2}$ hpi for 10 min and chased for up to $2\frac{1}{2}$ h: NP in cytoplasm.

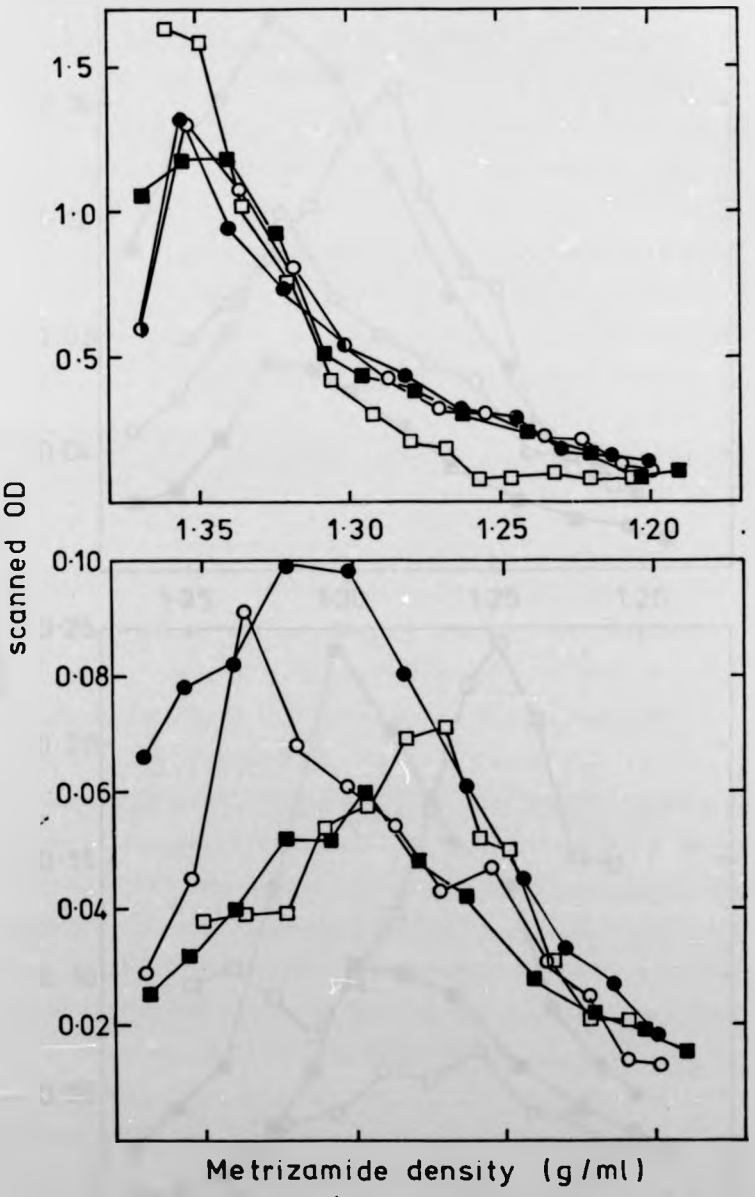


Fig. 3/11 Pulsed at $3\frac{1}{2}$ hpi for 10 min and chased for up to $2\frac{1}{2}$ h: M in cytoplasm.

- ○ 5 min chase
- ● 20 min chase
- □ 60 min chase
- ■ 150 min chase

Profiles obtained after Metrizamide centrifugation of infected cells.

Fig. 3/12 Pulsed at $3\frac{1}{2}$ hpi for 10 min and chased for up to $2\frac{1}{2}$ h. NS1 in cytoplasm.

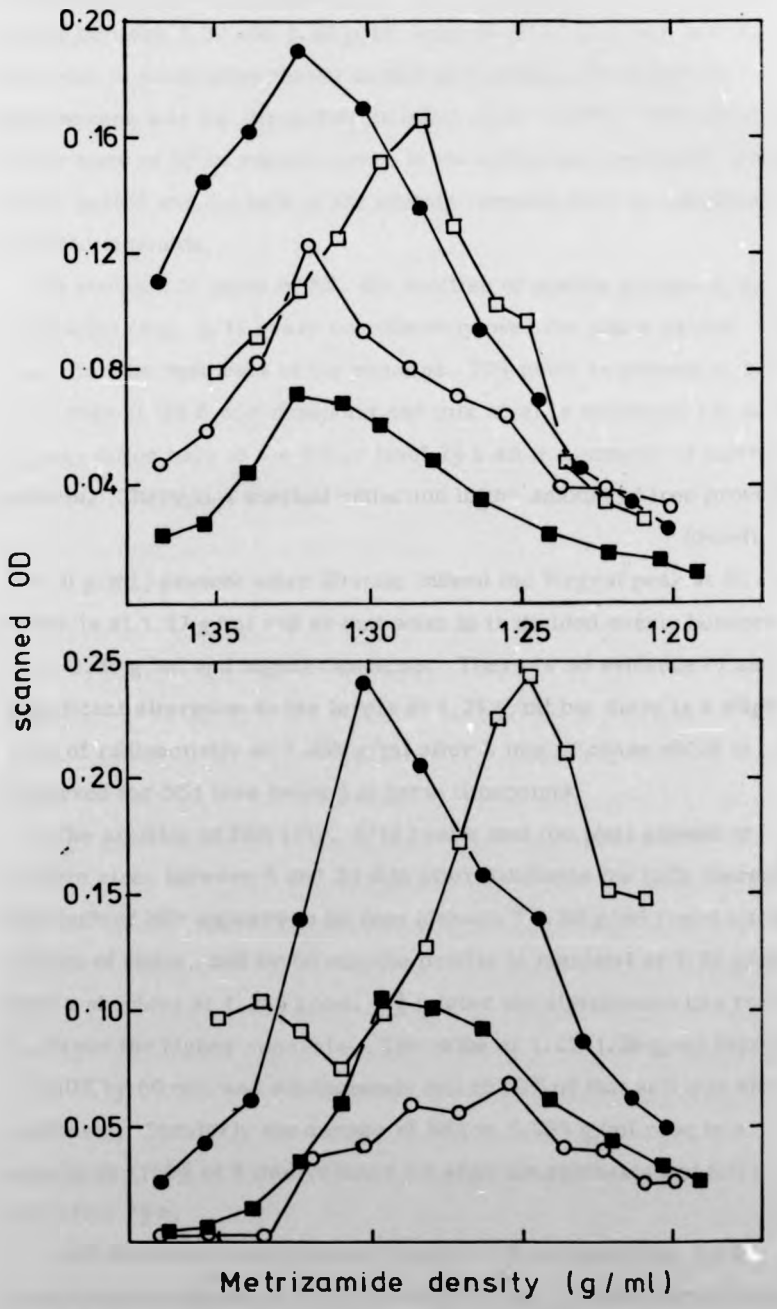


Fig. 3.13 Pulsed at $3\frac{1}{2}$ hpi for 10 min and chased for up to $2\frac{1}{2}$ h: HA2 in cytoplasm.

- ○ 5 min chase
- ● 20 min chase
- □ 60 min chase
- ■ 150 min chase

at the densities of the complexes. The difference in profile at all points between 1.30 and 1.20 g/ml suggest that this result may have been due to some abnormality in this particular culture and the phenomenon was not repeatable in other experiments. Thus the changes in the state of NP in substructures in the cytoplasm are small over the chase period and the bulk of the protein remains free or complexed with Metrizamide.

In contrast to those of NP, the profiles of matrix protein in the cytoplasm (Fig. 3/11) vary considerably over the chase period. 20 min after the synthesis of the proteins 70% more is present at 1.27 g/ml than at the 5 min timepoint,and this level is maintained at 60 min but has fallen back to the lower level $2\frac{1}{2}$ h after synthesis of matrix protein. protein. There is a marked reduction in the amount of free protein (density $\geq$ 1.30 g/ml) present after 20 min; indeed the largest peak at 60 min chase is at 1.27 g/ml and at that point M is divided evenly between 1.20-1.30 g/ml and higher densities. There is no evidence of any significant alteration in the levels at 1.24 g/ml but there is a slight peak of radioactivity at 1.255 g/ml after 5 min of chase which is also observed for NS1 (see below) at some timepoints.

The profiles of NS1 (Fig. 3/12) show that the total amount of protein rises between 5 and 20 min after synthesis but falls thereafter. The bulk of NS1 appears to be free (density $\geq$ 1.30 g/ml) until after 20 min of chase, and by 60 min the profile is maximal at 1.28 g/ml with a shoulder at 1.255 g/ml. $1\frac{1}{2}$ h later the distribution has returned to favour the higher densities. The value at 1.27-1.28 g/ml increased to 180% by 60 min and subsequently fell to 60% of that at 5 min after synthesis. Similarly the amount of NS1 at 1.255 g/ml rose to a maximum (160% of 5 min values) 1 h after its synthesis and fell to 50% after $2\frac{1}{2}$ h.

HA2 followed a rather more clearly defined path (Fig. 3/13). At 5 min after synthesis at $3\frac{1}{2}$ hpi there was little present in the cytoplasm but the amount rose to a maximum value 15 min later which was

maintained at 60 min after synthesis but fell considerably over the next $1\frac{1}{2}$ h. At 20 min after synthesis the bulk of HA2 appeared at 1.30 g/ml though there was a shoulder of activity at 1.255 g/ml. By 60 min of chase the peak had become shifted to this density with much less HA2 at higher densities. $1\frac{1}{2}$ h later the total amount of HA2 was much reduced and most of what was left was at the density of free, unglycosylated protein. Glycosylated proteins are thought to band at about 1.25 g/ml as does HA2 from disrupted virions (Section 3.2A), thus these profiles might illustrate the production of HA2, via cleavage, from the HA precursor, its glycosylation and finally loss from the cell, presumably in virions. In another experiment, it appeared that the virus growth cycle was retarded and a peak of HA2 was observed at a density characteristic of free virus (1.21 g/ml) after chasing for $2\frac{1}{2}$ h. This would appear to be at a stage between the 1 h and $2\frac{1}{2}$ h profiles illustrated in Fig. 3/13.

However, there is an alternative explanation arising from the observation of peaks of both M and NS1 at 1.255 g/ml, along with HA2. These three could thus be present in the form of a complex.

In the cytoplasm complexes at 1.27 g/ml and 1.255 g/ml but not 1.24 g/ml are tentatively suggested by these Metrizamide profiles. At 1.27 g/ml M and NS1 were present and the presence of NP cannot be excluded. The greatest amounts of M and NS1 appeared at this density 60 min after synthesis and these proteins decreased in amount in the following $1\frac{1}{2}$ h. There was no clear indication of a concentration of viral proteins at 1.24 g/ml as appeared in the nucleus but, unlike the nucleus, some suggestion of viral proteins M and NS1 was noted at 1.25 g/ml. HA2 also banded at this density but since the membrane bound glycosylated protein has a similar density this technique was unable to resolve unambiguously the state of HA2.

Discussion

Structures from disrupted virions resolved by Metrizamide

The Metrizamide gradient analysis revealed that all the disruption methods resulted in a considerable proportion of viral proteins banding between 1.25 and 1.29 g/ml, densities characteristic of protein rich RNP's. The principle uridine labelled RNA band was at 1.255 g/ml, in agreement with Hudson et al. (in press) which could be taken to be an NP containing RNP. RNP's at higher densities can be assumed to have a higher protein content (Rickwood and Birnie 1975) and the finding of M at these densities suggests that SVP's containing M protein must be produced. An SVP of this type has been described by Reginster and Nermut (1976) after fixing and subsequently disrupting spikeless particles whilst Skehel (1971) showed an association of M protein with SVP's produced by DOC treatment. Whether this is a stable structure and whether it is involved in the virus multiplication cycle in vivo has not been determined.

Skehel (1971) found that DOC treatment completely removed the glycoproteins and these were also absent from the cores of Reginster and Nermut (1976). The finding of HA2 concentrated at a density of 1.25 g/ml is consistent with these findings since free glycoproteins are found at these densities.

Structures from infected cells resolved by Metrizamide

Results have been presented which suggest the presence of structures containing viral proteins and,by implication, RNA in the nuclei of infected cells. It is possible to evolve a general plan of assembly and movement of these complexes which is consistent with these results. However, the evidence for these viral substructures is weak, and of its own account, does not justify more than tentative suggestions. With these provisos in mind, I shall bring together the salient features of this analysis.

In almost all instances the major proportion of viral proteins were found at densities > 1.29 g/ml. This is the density range in which free protein is found, but it is now clear that Metrizamide forms complexes with protein which band at densities from 1.29 to 1.50 g/ml (Rickwood et al., 1974). These are reversible interactions and variable in extent. It seems likely that interactions between other structures and Metrizamide are also possible which might lead them to exhibit a high and variable density. This would explain the absence of large concentrations of NP containing RNP's in the cytoplasm as discussed below. However, despite the variable profiles at high densities, regular profiles of viral proteins

in the density range 1.20-1.30g/ml were obtained. These profiles might possibly represent viral structures found in the infected cell, though their quantitation, especially with respect to free proteins, is not reliable.

In the studies reported above, there were three densities exhibiting a concentration of viral proteins and it is on the basis solely of comigration on Metrizamide gradient that the existence of particular structures is inferred. Extensive further analysis including, for example, velocity centrifugation is required to confirm that these proteins are in a single structure. The three densities are 1.27, 1.25 and 1.23 g/ml. The former is indicative of a protein rich RNP since free protein bands at 1.29 g/ml and above, whilst free vRNA is found to band at 1.15 g/ml (Hudson et al., in press) and complexes containing differing proportions of RNA and protein band in between. The major RNP of the virus is found at 1.255 g/ml and the structure observed in gradients from infected cell extracts might be similar. However, both structures incorporating membranes and free glycoproteins band at this density. The 1.23 g/ml fraction might represent a protein deficient vRNP although 1.23 g/ml is close to the density of a chromatin band found in this and other studies (Birnie et al., 1973; Rickwood et al., 1974) and might indicate the association of free viral protein or a structure containing viral proteins with the cell DNA.

The Appearance of SVP's in the Nuclei of Infected Cells: 1.27 g/ml

After a pulse at 2 hpi, NP and NS1 are clearly resolved in peaks which co-run at 1.27 g/ml and chase out by 5 hpi. Synthesis of viral proteins at 5 hpi leads to a similar coincidence of viral protein peaks but M is also apparent. After synthesis at 3½ hpi, NP and M appear to chase into this putative SVP up to 20 min. By 60 min this accumulation is no longer apparent. NS1 also appears at this density, but peaks at 60 min and is not apparent after a 2½ h chase. It is a structure of this density which would form a likely candidate for the M-containing core postulated in Section 2, since it contains M, NP and RNA, appears rapidly (within 15 min), and then disappears. However, NS1 is also present at this density. If this is the putative core structure, several explanations are possible:
(1) NS1 may be utilized in assembling the SVP, only to be left within the nucleus when the particle is transported out. The kinetics of NS1 accumulation at this density are different from those of M and NP.

(2) There may be separate structures of NP, M and RNA and NP, NS1 and vRNA. This explanation is attractive as it is consistent with the different kinetics of NS1 accumulation and accounts for the absence of a shift in density which would be expected if NS1 became associated with a pre-exisiting complex of M and NP. It is also consistent with the appearance of NP and NS1 at this density at 2 hpi when the synthesis of M is too low to be detectable.

This is, however, no more than a preliminary and tentative suggestion that such a complex may exist in the infected cell. There is no direct evidence that NP and NS1 or NP and M are present at this density in single structures. There is no direct evidence that RNA is present in these putative SVP's and none that it is vRNA. The evidence that any such SVP's are involved in the infection cycle is circumstantial and there is no quantitative basis for their role.

The Appearance of Viral Proteins in the Nuclei of Infected Cells at 1.23 g/ml

There was no indication of an accumulation of viral proteins at at 1.23 g/ml 2 h after infection. At $3\frac{1}{2}$ hpi NP and M were present at 5 and 20 min after synthesis but levels had fallen away 40 min later. NS1 was not observed until 20 min after synthesis at 5 hpi and appeared together with NP and M. The relevance of these observations is not clear. Again, if this accumulation is caused by a structure, it appears to chase away between 20 and 60 min after its assembly. It might be caused by vRNP's containing an excess of RNA or possibly due to an association of viral proteins or viral substructures with chromatin which gains circumstantial support from the absence of such a structure in the cytoplasm. Further characterization on velocity gradients would determine whether it was a single structure, but the amount present was too small for such an analysis.

The Appearance of Viral Proteins in the Cytoplasm of Infected Cells

The Metrizamide studies were extended to the cytoplasm for the $3\frac{1}{2}$ hpi set of experiments in an attempt to follow putative vRNP's into this compartment. However, the complexity of structures present in the cytoplasm with densities in the range of interest rendered interpretation very difficult. The problem of variable density of membranes and the position of polysomes and ribosomes all complicated the overall picture.

The profiles of NP were remarkably similar for all the chase times examined after $3\frac{1}{2}$ hpi. This is consistent with the theory that SVP structures may be rapidly transported from nucleus to plasma membrane and incorporated into virions. However, there is considerable evidence for NP containing RNP structures from sucrose gradient analyses of the cytoplasm (Compans,1973;Klenk et al.,1974; Hay,1974; Krug, 1971,1972). This suggests that the absence of such structures in this study arises from some other cause of which the most likely is complexing with Metrizamide resulting in an increase in apparent density.

NS1 shows predominantly at densities >1.29 g/ml which may reflect its association with ribosomes (1.305 g/ml) and polysomes (1.350) but might also reflect complexing with Metrizamide. At lower densities, a peak of NS1 at 1.28 g/ml developed by 60 min, and a shoulder at 1.255 g/ml which was reduced by 150 min, whilst the profile of M indicated similar accumulations. No accumulation at 1.23 g/ml like that found in nuclei was observed. It is difficult to interpret the relevance of these observations due to the low level of M and NS1 present in the cytoplasm, the apparent lack of involvement of NP and the complexities of structures in the cytoplasm. It is possible that a1.27 g/ml complex of M and NS1 is present at 20 to 60 min after the synthesis of these proteins and this might have originated in the nucleus. There is no evidence for NP at this density which, together with the Results of Section 2, suggest that this is not the case.

SVP's in Infected Cells

Studies in this area are hampered by the lack of a method of sufficient unambiguous and / resolution and the complexity of host components in the cell. The cytoplasmic studies using sucrose gradients generally report NP and sometimes the P proteins at an intermediate density amongst the cytoplasmic fractions and it is suggested that this represents SVP's containing RNA sedimenting freely at their density rather than in association with any cellular structures (Compans, 1973; Meier-Ewert and Compans, 1974; Klenk et al., 1974; Hay, 1974). Analysis of RNP's from disrupted virions on sucrose gradients has shown them to be heterogeneous (Duesberg, 1969; Kingsbury and Webster, 1969; Pons, 1971), but studies of viral RNP structures in infected cells have not led to any clear pattern of behaviour. RNP's have been observed in both nucleus and cytoplasm (Pons, 1971; Krug 1971, 1972) and a substantial proportion of cytoplasmic RNP's contain cRNA. Pons (1972) found that polysomal viral RNA was complementary to the virion and was present in RNP's which contained both NP and NS1. The large amount of NS1 and NP in the polysomal density (1.35 g/ml) may represent these RNP's. Krug (1972) was drawn to the conclusion that vRNP's in the nucleus did move into the cytoplasm, which is in agreement with the appearance of a putative complex at 1.27 g/ml in this study in both nucleus and cytoplasm together. Assadullaeff et al. (1975) reported a 120 S polymerase complex in the nucleoplasm of infected cells which contained NP. The complex functioned late in infection and was insensitive to AMD, from which they deduced that it was synthesizing vRNA. They tentatively suggested that this might chase into the cytoplasm and break up into the 50-60 S structures which corresponded to virion RNP's, all of which contained NP. The 120 S structure might correspond to the 1.27 g/ml accumulation observed in the present study, whilst the 1.255 g/ml peak in the cytoplasm might be the 50 S complex since both exhibit some similarities with virion RNP's. However, the results of Assadullaeff et al. are equivocal and they did little to characterize their structures. It is not clear, for example, whether the complexes contain v or cRNA.

It is a common difficulty in reviewing studies of this sort that comparisons between them are rendered almost impossible by different methods of analysis. The behaviour of RNA/protein complexes in velocity gradients after fixing (Krug, 1972; Assadullaeff et al., 1975) and unfixed (Pons, 1972), in equilibrium gradients of glycerol (Pons, 1972), caesium chloride (Krug, 1972; Assadullaeff et al., 1975) and Metrizamide give rise to data which cannot easily be co-ordinated.

5. Conclusion

The results of this study indicate tentatively that viral RNP complexes containing M as well as NP may be resolved by disrupting virions, and that viral RNP complexes containing NP, M and/or NS1 may be resolved from infected cells. The relevance of these putative SVP's to the virus growth cycle is not clear, though their appearance is in basic agreement with the movement of viral components suggested in Section 2.

However, this study shows that Metrizamide analysis of infected cells is complicated by incomplete separation of components, by cosedimenting of different structures (such as glycoproteins and viral RNP's of 1.25 g/ml) and by variable association of proteins with Metrizamide itself, leading to complexes at higher densities. Though the technique can demonstrate the existence of such SVP's, it is unable to reliably quantify them, especially with respect to their content of viral proteins. The technique, as applied in this study, has failed to resolve unambiguously the questions posed of it.

APPENDIX

1. Introduction

The use of isolated subcellular fractions has been invaluable in the understanding of the mechanism of function of eukaryotic cells. The distribution of molecular species between cell compartments, measured after separation by subcellular fractionation techniques, has thrown light on the function and roles of these species. At very least it provides a basis for the study of the location and movement of the entity under investigation, about which further theories can be critically examined and tested. Subcellular organelles have been used to study particular areas of cell metabolism in isolation such as mitochondria for oxidative phosphorylation and fatty acid oxidation, nuclei for replication of DNA, transcription and modification of RNA.

Cell fractionation has brought to virology the ability to devise a scheme of virus infection of the host cell around which function and then control of viral components can be elucidated. Cell fractionation to produce nuclei is of great potential value in studying the multiplication of influenza virus since this has an absolute requirement for a functional nucleus (Barry et al., 1962; Kelly and Dimmock, 1974; Follett et al., 1974). The isolation of this organelle and the analysis of the viral components which are transported there and may subsequently return to the cytoplasm are key features in the understanding of the nuclear requirement.

Cell fractionation has already highlighted several features of the influenza virus growth cycle. A few examples will illustrate this point. Input RNA rapidly associated with the nucleus after infection (Stephenson and Dimmock, 1975) as do newly synthesized NP, M, NS1 (Taylor, Hampson and Wnite, 1969; Lazarowitz et al., 1971) though they have not been observed to return to the cytoplasm (Hay and Skehel, 1975; Krug and Etkind, 1973).

A. Problems of Cell Fractionation

Turning to the methods of subcellular fractionation used to isolate nuclei, three problems can be defined. The first is homogeneity of the cells from the initial sample. In the classical fractionation systems, liver was the most widely studied tissue. This consists of a number of different cell types each of which has a different function and morphology and may give rise to a heterogeneous response to steps in the fractionation procedure. The problem of homogeneity is of much less consequence in the fractionation of cells in continuous culture since they are derived from a clone, though in an unsynchronized population there will be differences according to the state of the cell cycle. However, cellular heterogeneity remains a factor in the fractionation of primary cells in culture such as the chick embryo fibroblasts used in this study.

The second problem encountered in isolating nuclei is to determine the yield of the fraction and to minimize loss of nuclear components. Though large structures such as the chromatin are likely to remain in the organelle even after considerable disruption of its membranes, some components, particularly those soluble in the nucleoplasm, may be readily leached out of the nucleus.

Contamination of the nuclear fraction with material from other compartments within the cell forms another major problem. This can take the form of reassortment of components from the soluble fractions contained within other compartments of the cell. It is conceivable that they gain entry into the nuclei via damaged or 'leaky' nuclear membranes. Contamination can also be caused by the incomplete removal of adjacent fractions such as the endoplasmic reticulum which forms a contiguous layer with the outer membrane of the nucleus. Finally the nuclear fraction may contain impurities which have become associated due to specific or non-specific attachment to the nuclear membrane, especially when the nuclei become 'sticky' after removal of the outer membrane and tend to aggregate. Contaminants may adhere directly to the exposed membrane or may become trapped amongst aggregates of nuclei.

B. Review of Nuclear Fractionation Development

Bearing these problems in mind I will review a brief selection of techniques which have been used for the isolation of nuclei outlining the development of a rationale to overcome these problems. The basic classical fractionation as published by Dounce (1943) and subsequently modified by others comprises an initial disruption of the cells, low speed centrifugation to separate out the nuclei and a variety of washing procedures to remove contaminants.

Perhaps the most critical feature of the fractionation is the initial homogenization of the cells. The membrane disruption must be severe enough to liberate the nuclei without being violent enough to collapse them. For studies on animal tissues the Waring blender and Potter-Elvehjem homogenizer are favoured instruments, though for tissue culture cells a simple Dounce homogenizer is widely used. However, the shearing force actually applied to the cells by these instruments is impossible to calibrate accurately. Thus this step in the procedure must be adjusted by trial and error to the particular instrument, operator and tissue involved. On the other hand, the homogenization medium is amply documented. Dounce initially developed an acid medium using citric acid at pH 6 (Dounce, 1943) and discovered that as the pH was lowered so the purity of the nuclei increased to limits such that Higashi et al. (1966) used pH 2.5-2.8 in the isolation of nuclei from a Walker tumour. However, this harsh treatment does have a serious effect on the composition of the nucleus, for example, histones are removed from rat liver DNA in nuclear preparations (Dounce et al., 1966), and limits the possibility of enzyme analysis and study. These extractions were performed in hypotonic buffer, but isotonic media (usually 0.25 M sucrose) have also been employed to reduce the damage to nuclear membranes and consequent loss of contents (Schneider, 1948; Hogeboom et al., 1952; Blobel and Potter, 1966).

Fractionation of animal tissues usually employs filtration through gauze, flannelette, cheesecloth or glasswool after disruption to remove fibres and undisrupted clumps of cells. Following homogenization, the

nuclei are separated from the bulk of cellular debris by a centrifugation, typically of 300-700 g for 5-10 min. The pellet is resuspended and the nuclei subjected to procedures designed to remove contaminating species. In the early experiments Dounce centrifuged the nuclei out of water. Later isotonic sucrose became popular (Schneider, 1948) and centrifugation through a sucrose interface (typically 0.25 M to 1.9 M) to 'jolt' the nuclei clean was also attempted (Maggio et al., 1963; Kodama and Tedeschi, 1963). The method of Chauveau et al. (1956) consisted simply of homogenizing the tissue in 2.2 M sucrose and centrifuging this at 40,000 g for 60 min to pellet the nuclei. This gave a low yield of quite pure nuclei. However, the control of temperature and concentration of sucrose are critical since large changes in viscosity of sucrose at these high concentrations occur with small fluctuations of temperature. Centrifugation on linear sucrose gradients (11-40% w/w) has also been used to separate whole cells (which pellet) from nuclei which move as a band a short way into the gradient after a spin of 300 g for 4 min.

Following the studies of Schneider and Petermann (1950) it became general practice to use low concentrations of divalent cations which helped to reduce the fragmentation of nuclei and reduced the amount of clumping.

Neutral detergent treatments have been used in several methods (for example Hubert et al., 1962; Rappoport et al., 1963). These solubilize most of the cytoplasmic membrane systems including the endoplasmic reticulum adjacent to the nuclei (Holtzman et al., 1966) and thus obviate the need for dense sucrose solutions. The treatment is drastic, though no more so than the dilute acid procedures, and may result in damage to nuclear membranes and leaching of contents. The integrity of cytoplasmic components is, of course, lost and the activities of enzymes sometimes affected.

Nuclei have also been prepared using organic solvents (Dounce et al., 1950) in which the homogenate is subjected to a series of sedimentations in and flotations on mixtures of benzene and carbon tetrachloride. Although Georgiev (1967) concluded that this method preserved the full complement of soluble proteins in the nucleus, there are doubts about

the results obtained due to the profound effect of the solvent on the morphology of the structural components of the nucleus.

Zonal centrifugation has been used to separate different types of nuclei from rat liver (Johnston et al., 1968). This separation is on the basis of DNA content and morphology and can provide separation of parenchymal from stromal nuclei from liver tissues. This technique has also been used to separate nuclei from cellular contamination. In one study (El-Aaser et al., 1966) four fractions consisting of vesicles, mitochondria, plasma membranes and nuclei were separated from a crude nuclear preparation.

Recent attempts to tackle the problem of cellular homogeneity have used zonal centrifugation which has provided the resolution to isolate nuclei with different ploidy within the population.

C. Characterization of Nuclear Fractions: Yield

Yield of nuclei is most readily measured by counting the nuclei in a defined volume under phase-contrast microscopy. This does, however, give little indication of the integrity of the nuclei, and suffers the severe drawback that any aggregation of nuclei (such as frequently occurs in standard fractionation techniques involving detergents) precludes its use. Chemical analyses commonly use the measurement of DNA to represent nuclei though /protein and total nitrogen have been used (Roodyn, 1972). DNA determination has the advantage over nuclear marker enzymes, like NAD pyrophosphorylase (Hogeboom and Schneider, 1952) in being in large conformations which are less likely to be leached out of nuclei, though this could also be construed as a disadvantage in producing over-optimistic values for the yield of complete nuclei. The problem of homogeneity of nuclei with respect to their position in the cell cycle can be assessed by comparison of the yields of DNA given from recovery of incorporated [^{3}H]-thymidine with the chemical measurement of total DNA content. A difference in the behaviour of these two markers might indicate alteration of the fractionation properties of newly divided nuclei (measured by radiolabel incorporation) over the total population of nuclei (by chemical

analysis). DNA polymerase activity is another potential nuclear marker activity, but the enzyme is considerably affected by prior treatment to the cells (Weser/ and Bischoff, 1970) and by the nature of the isolation medium (Waqar et al., 1971) whilst there is some controversy over its location solely in the nucleus (Friedman, 1970; Lindsay et al., 1970). The activity of RNA polymerase suffers similar drawbacks as a criterion of nuclear purity (Moulé, 1970) since it is readily solubilized (Liao et al., 1968).

A more refined test of nuclear integrity by Ord and Bell (1970) tested nuclear isolation media by transplanting the resulting nuclei into amoebae and observing their survival. The ability to restore the viability of an enucleate cell is a most sensitive test of nuclear function. Incidentally, the results of Ord and Bell suggested that viability of the nuclei was rapidly lost following a few minutes' exposure to isolation media, illustrating the value of very rapid methods of nuclear preparation.

The present study relied on assays of DNA to provide a value for the yield of nuclei. The chemical assay of DNA in the fractionation was reinforced by the counting of subcellular fractions prepared from cells prelabelled with [^{3}H]-thymidine. Counting nuclei in a haemocytometer was abandoned because of problems of aggregation, whilst DNA and RNA polymerase assays were not used for reasons described above. The difficulty of the technique of nuclear transplantation rendered it outside the scope of the present study.

Characterization of Nuclear Fractions: Contamination by Endoplasmic Reticulum

Contamination of nuclei by endoplasmic reticulum is likely since the outer nuclear membrane is contiguous with cytoplasmic membranes. Unfortunately there are very few enzymes specific for this fraction. Glucose-6-phosphatase has been widely used (Wallach and Lin, 1973), though its reactivity in some cell lines is low. NADH-oxidases are often used as markers for endoplamic reticulum, although there is some doubt over their unique specificity for this compartment. NADPH-

cytochrome c reductase has been shown to be located in endoplasmic reticulum by Phillips and Langdon (1962), and was successfully employed as a marker in CEF fractionation by Bingham and Burke (1972). In influenza virus infected cells the viral haemagglutinin has been shown to concentrate around the nuclear periphery (Breitenfeld and Schafer, 1957; Kelly and Dimmock, 1974) and has also been used to indicate cytoplasmic contamination of nuclear preparations (Stephenson and Dimmock, 1974; Hudson and Dimmock, 1977).

In the characterization to be described NADPH-cytochrome c reductase was used to estimate contamination by endoplasmic reticulum since NADH oxidases appeared to be less specific markers. The former enzyme also had the advantage over glucose-6-phosphatase that its use in CEF cells was already documented.

A further rigorous test of cytoplasmic contamination involving the measurement of viral haemagglutinin levels was employed for the reasons just described.

Characterization of Nuclear Fractions: Contamination by Mitochondria

The presence of mitochondria has been estimated by enzyme assays measuring the amount of choline dehydrogenase, cytochrome oxidase, monoamine oxidases, succinate dehydrogenase and glutamine dehydrogenase among others (Wallach and Lin, 1973). Succinate dehydrogenase was chosen for this study, since it is assayed by a simple, rapid and accurate method which had been successfully applied to the fractionation of CEF cells (Bingham and Burke, 1972).

Plasma Membrane Contamination

Plasma membranes are the most likely contaminant of nuclear preparations (Zentgraf et al., 1969; Durham et al., 1975; Deduve and Berthet, 1954; Hogeboom and Schneider, 1952; Song and Bodansky, 1967) and a number of methods to assess their presence have been described (for review:Wallach and Lin, 1973). Methods involving the tagging of plasma membranes of intact cells covalently with labelled probes can be divided into three major groups.

(1) Low molecular weight labels, such as [^{35}S]-formylmethionyl-sulphone methyl phosphate (FMSMP) (Bretscher, 1971).

(2) Large molecules like [^{203}Hg]-labelled N-(3-mercuri5methoxypropyl) poly-D, L alanyl amide (an SH blocker).

(3) Enzymatic iodination techniques in which lactoperoxidase catalyses the iodination of exposed tyrosine residues in the presence of hydrogen peroxide (Phillips and Morrison, 1971).

Unfortunately the small molecular weight probes were found attached to the haemaglobin of intact erythrocytes in significant amounts (15% for FMSMP) and thus had penetrated into the cell. The large molecular weight tags may be an improvement, though many are polydisperse with the possibility that the smaller ones may too be able to enter the cell. There are also problems with the lactoperoxidase method, since an activated iodine species was discovered in the reaction. It was thought that this would penetrate the membrane, but the results with erythrocytes show only 3% of the label attached to haemaglobin.

Alternatively enzyme activities found in plasma membrane fractions can be used as markers. These include 5' nucleotidase (Coleman and Finean, 1967; de Pierre and Karnovsky, 1973), Na^+-K^+ stimulated ATPase, alkaline phosphatase, phosphodiesterase and adenylate cyclase (for example, Bosmann et al., 1968; Durham et al., 1975). Of the above group of enzymes, Bosmann et al. found 5' nucleotidase to be the most highly concentrated in plasma membranes, though de Pierre and Karnovsky reported some association with other cell components. The Na^+-K^+ stimulated ATPase could be concentrated 30-fold, during purification of the plasma membrane (Bosmann et al., 1968), but, in the work on CEF cells by Bingham and Burke (1972) this activity was too low to be of use. Alkaline phosphatase comprises a group of enzymes and thus appears to be less satisfactory as a cell component marker. Phosphodiesterase and adenylate cyclase are both specific enzyme activities and both have been found on plasma membrane fractions (Bosmann et al., 1968; Durham et al., 1975).

From these enzymes 5' nucleotidase and adenylate cyclase were selected to represent plasma membrane contamination in the present study. 5' nucleotidase is an extremely active enzyme relatively unaffected by membrane solubilization treatments and a much used marker for plasma membranes. Adenylate cyclase was chosen due to the very sensitive assay available for its detection (Salomon, Londos and Rodbell, 1974) and its high specificity for plasma membrane (Durham et al., 1975; Davoren and Sutherland, 1963). In addition, these enzymes are thought to be located on opposite sides of the plasma membrane, the catalytic unit of adenylate cyclase being internal (Perkins, 1973), whilst 5' nucleotidase is an ectoenzyme (Newby, Luzio and Hales, 1974).

D. Analysis of Data

In the present study, exclusive use has been made of the rigorous analysis provided by the "balance sheet" approach, in which yields and contaminants are expressed as a proportion of the total recovered or of the starting homogenate rather than as a specific activity, following the recommendation of Wallach and Lin (1973). This reveals activation and inhibition of marker enzymes, interference with assay procedures or differential quenching of radiolabel during fractionation. In the analysis of a complex organelle such as the nucleus, the optimization of specific activity will favour procedures which leach out genuine nuclear proteins whilst the apparent depletion of negative markers will be deceptively high due to the large content of protein in the nucleus.

E. Characterization of Fractionation Methods Used in the Study of Influenza Virus Replication

In the field of influenza virus replication, considerable use has been made of subcellular fractionation to isolate nuclei. However, the methods used to characterize these nuclei have been far from comprehensive.

The first studies on influenza virus replication in which isolated nuclei were obtained from infected cells was by Taylor et al., 1969; 1970). This group produced a crude nuclear fraction for which no characterisation is mentioned, but after further purification steps they

quantitated the yield from recovery of incorporated [^{3}H]-thymidine from a 48 h prelabel. Krug (1971) and Lazarowitz et al. (1971) used electron micrographs of thin sections of their nuclear preparations to determine the absence of cytoplasmic tags whilst Krug reported that no attempt was made to measure any leaching of nuclear contents into the cytoplasm.

No mention was made of any checks on the yield or contamination of the nuclei obtained by Mahy et al. (1975) or Gregoriades (1973). Hay (1974) used some enzyme assays and did find that further purification of nuclei did not alter the viral protein ratios, whilst Gregoriades claimed that the "[viral] nuclear protein is too great in amount to be explained by cytoplasmic contamination". Krug in later papers (Krug, 1972; Krug and Etkind, 1973) detailed no other checks though he found considerable problems with so-called cytoplasmic viral contaminants in the isolated nuclei and then leaching out of apparently nuclear viral components on more extensive detergent treatments. Krug and Soeiro (1975) further developed the fractionation into subnuclear fractions and characterized the nucleoplasm as containing prelabelled RNA's of > 45 S, whilst the nucleoli contain precursor rRNA's of 32-45 S. Stephenson and Dimmock (1975) used the absence of cytoplasmic tags attached to their nuclei when viewed under phase-contrast microscopy and the absence of virus haemagglutinin associated with the nuclear fractions as a measure of contamination whilst a [^{3}H]-thymidine prelabel measured the yield of nuclei.

Although no mention of criteria of nuclear yield and purity in some of the above reports does not necessarily indicate that no checks were performed, it does suggest that such tests are not considered important in these fractionation experiments. The unquestioning allotment of viral components to nuclear and cytoplasmic compartments according to precariously established dogma, together with the dismissal of their own data as leaching or contamination (Krug and Etkind, 1973) without consideration of any other alternative is indefensible.

Though each of the described methods of characterisation is valid, each also suffers drawbacks. Of the methods involving visualization of the nuclei, it can be argued that, if biochemical questions of location are to be asked, then biochemical criteria of purity should be applied. After all, contaminating cytoplasmic molecules cannot be seen, even in electron micrographs ! Furthermore no group has described the use of both methods simultaneously - low power analysis of large fields of nuclei by light microscopy for uniformity of preparation together with high resolution analysis by electron microscopy of 10-20 nuclei for more rigorous exclusion of cytoplasmic tags. Analysis of yield by distribution of [^{3}H]-thymidine incorporated during a prelabel is perhaps justifiable as a single marker, though there is the possibility of selecting a population of cells which may not be representative of the whole culture, but none of the groups using this criterion (with the exception of Stephenson and Dimmock) relate it to any measure of contamination.

Some workers performed mixing experiments in which the species under investigations was added to a homogenate immediately prior to fractionation or at some stage in the procedure to assess how much artefactual association or reassociation may have taken place. However, the selection of these controls is fraught with hazard due to the difficulty of mimicking the situation in the fractionation system. For example, the addition of a suspected contaminant to a cell homogenate and subsequent reconstruction only tests whether the contaminant is redistributed during extraction, whilst the perturbing process which associates it with the nucleus may, in fact, be the homogenization. The analysis of redistribution of a component from the plasma membranes, obtained in the detergent washes of a purification technique, can be performed by re-extracting it with unlabelled homogenate. However, this is unlikely to mimic the real situation since the membranes containing the species are likely to be highly perturbed by the initial treatment, and its transfer to other compartments either facilitated (by partial detergent solubilization) or retarded (by membrane vesiculation).

The validity of a comparison with a known cytoplasmic virus such as Newcastle Disease Virus or Semliki Forest Virus is debatable since a virus which can replicate successfully in enucleate cells may also accumulate components and even replicate in the nucleus of a nucleate cell.

F. Fractionation Techniques of this Study

Nuclei were isolated by two quite different methods. One was a conventional fractionation (Stephenson, 1974) utilizing homogenization by a stainless steel dounce machined to precise tolerance to maximise the reproducibility of this technique. The homogenization buffer was hypotonic and the cells were left to swell in this medium prior to the initial disruption. Following this treatment, the crude nuclei were always maintained in isotonic sucrose and all buffers contained Mg^{2+} to maintain the integrity of the nuclei. Repeated gentle treatment with the homogenizer produced a high yield of nuclei and helped to prevent clumping and aggregation of the crude nuclei during resuspension after each pelleting. Finally, the nuclei were twice washed with buffer containing a low concentration of a non-ionic detergent. This solubilized cytoplasmic structures, removed contaminating membranes from the nuclei and stripped off the outer nuclear membrane.

The second method used in this study was a novel technique which has recently been developed (Tsai and Green, 1973). The nuclear monolayer technique (NML) involved treatment of cell monolayers with a high (1-2%) concentration of non-ionic detergents. This solubilizes most of the cytoplasmic membranes and removes the majority of the cell cytoplasm. The nuclei remain fixed to the plate, presumably by a small amount of cytoplasm remaining beneath, and hence the term 'nuclear monolayer'.

The first fractionation method presented the opportunity for an exercise of the methods of characterisation reviewed above. It embodied many of the features developed over the past three decades to minimize the contamination and maximize the yield of nuclei, and it had already demonstrated differences in the cytoplasmic and nuclear fractions from influenza virus infected cells (Stephenson, 1974; Stephenson and Dimmock, 1974; 1975). The nuclear monolayer method was developed for use in the influenza CEF system during the period of this study (Hudson and Dimmock, 1977) and although this method did not lend itself to the characterization techniques reviewed and applied to the first method, it offered crucial advantages over the conventional fractionation.

The NML technique takes only minutes to perform compared with 60-90 min by the conventional method, and concentrated fractions can readily be obtained. This speed of preparation minimizes the natural movement of viral components during the fractionation period which can occur even at 4^{o} (see section 1; Stephenson and Dimmock, 1975). It reduces the risk of leaching nuclear components from the nucleoplasm and the artefactual reassociation of soluble components with the nuclei of cells, whilst the absence of centrifugation avoids "trapping" cytoplasmic entities amongst aggregates of nuclei. Its disadvantages lie in the small amount of cytoplasm ($\leq 10\%$) that remains beneath the nuclei and hence appears in the nuclear fraction and the denaturing effect of the high concentration of detergent on enzyme activities which obviates its characterization by the methods described above. Such characterization that is possible in these conditions has shown that 86% of prelabel [^{3}H]-thymidine is found in the nucleus compared with 84% by conventional fractionation and 92% of chased prelabel [^{3}H]-uridine is cytoplasmic compared with 90% for the CF (Hudson and Dimmock, 1977).

The rationale of the following exercise on the characterization of a nuclear fractionation technique rests not on the superiority of any of the individual parameters of characterization over those which have been employed previously, but rather that a more comprehensive survey has been used to calculate a numerical estimate of purity. Not only have specific positive markers for the nuclei been employed, but also the depletion of a variety of markers characteristic of other subcellular organelles (negative markers) has been catalogued. This information should provide a more rigorous definition of the nuclear fraction of the conventional fractionation method.

2. RESULTS

A. Justification of Methods Used in Characterization

(a) 5' Nucleotidase activity

The assay for 5' nucleotidase measures tritiated adenosine formed from [^{3}H]-AMP substrate by the enzyme. The reaction is stopped by the

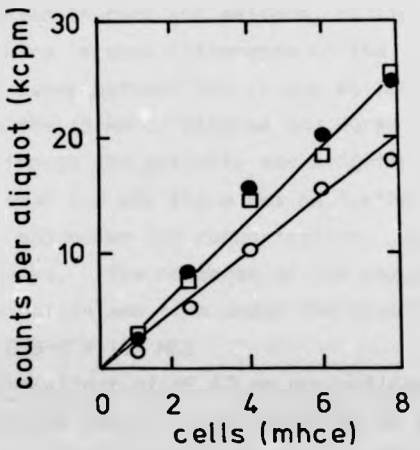


Fig. A/1 The magnesium ion-dependence of 5'nucleotidase. Aliquots of sonicated whole cell preparations were measured for 5'nucleotidase activity in the presence of ○: 0.18 mM Mg^{2+}; ●: 1.8 mM Mg^{2+}; □: 18 mM Mg^{2+}

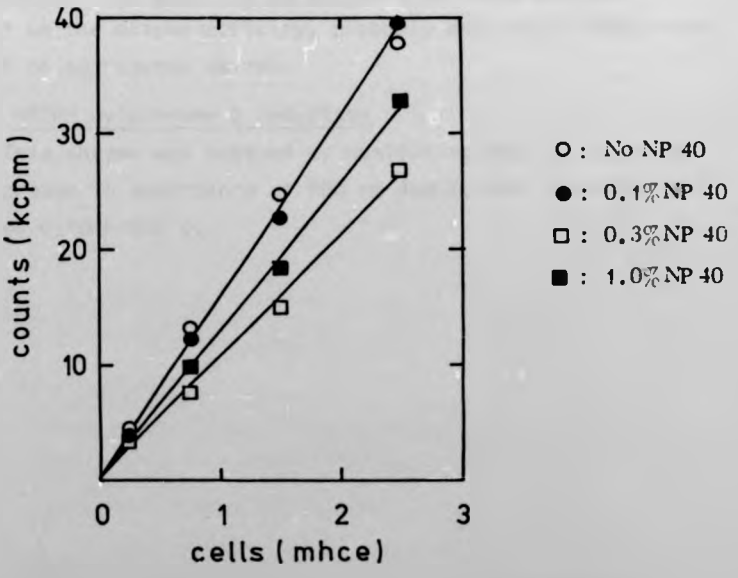


Fig. A/2 The effect of NP 40 on 5'nucleotidease activity

addition of barium hydroxide and magnesium sulphate, forming barium sulphate which also precipitates unreacted AMP, leaving the radioactive adenosine in the supernantant, which can then be counted (Avruch and Wallach, 1971).

There is some difference in the quoted Mg^{2+} concentration in the assay between Avruch and Wallach (1971) who used 0.18 mM and the 18 mM of Bingham and Burke (1972). Fig. A/1 shows that, though the activity was slightly reduced at 0.18 mM (78%) over 1.8 mM, there was no further stimulation at a 10-fold higher magnesium ion concentration. Thus 1.8 mM was used in all assays. The response of the enzyme was linear to both concentration and time under the conditions chosen over the range 0.3-4 x 10^6 HCE

The effect of NP 40 on nucleotidase activity

As the non-ionic detergent NP 40 was utilized in preparing the nuclear fraction, it was necessary to determine its effect, if any, on 5' nucleotidase activity. Fig. A/2 shows a typical experiment. 0.1% NP 40 had no effect and though 0.3% reduced the rate somewhat, 1% appeared less inhibitory. In general, NP 40 had a variable but small effect on the enzyme activity, probably due to its dispersive effect on aggregated matter.

(6) NADPH cytochrome c reductase

This enzyme was assayed by monitoring the initial rate of increase in absorbance at 550 nm due to the formation of reduced cytochrome c.

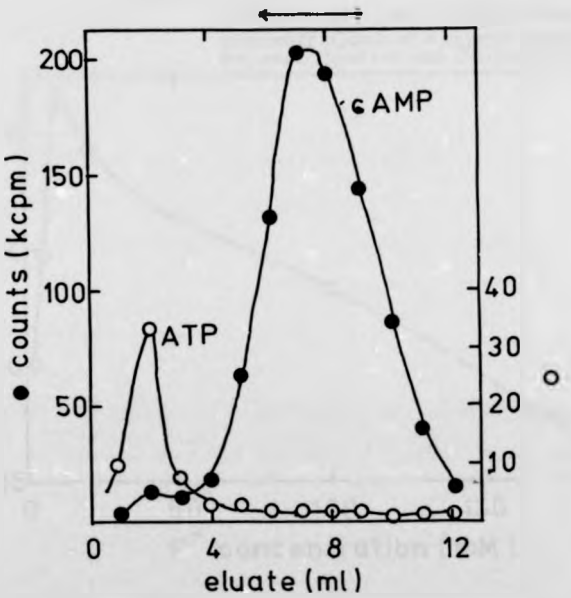


Fig. A/4 Elution profile of cAMP and ATP from Dowex column
⟷ represents assay sample.

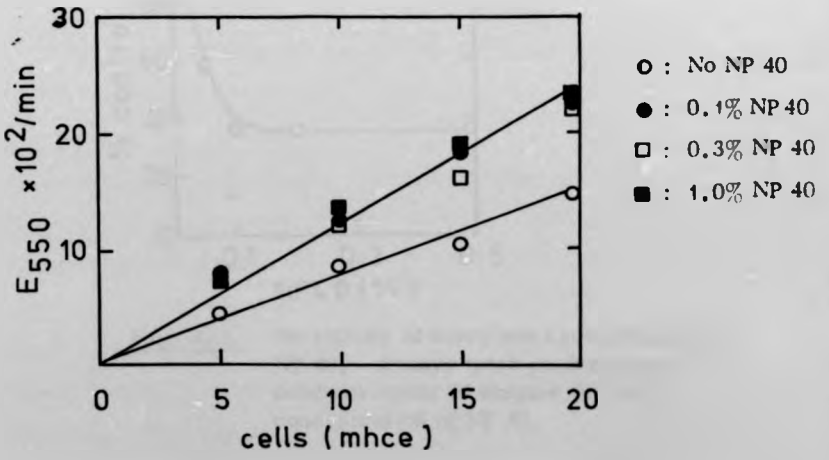
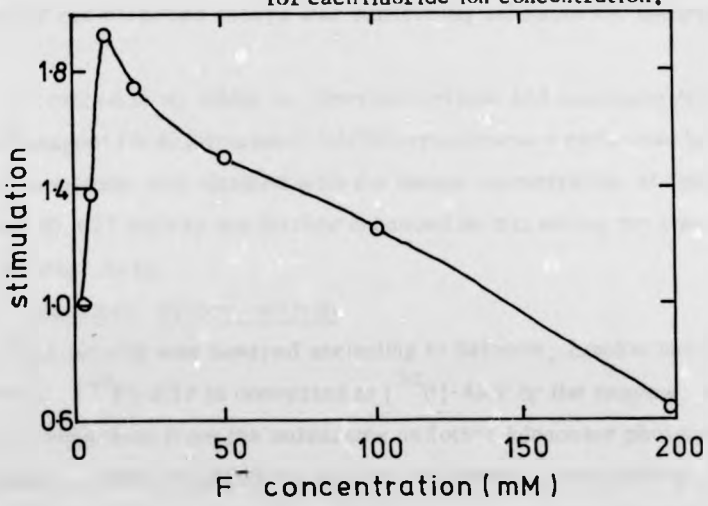


Fig. A/3 Effect of NP 40 on NADPH-cytochrome c reductase activity.

Fig. A/5 Optimum fluoride ion concentration for adenylate cyclase activity. Assays were performed on concentrations of enzyme between 1-10 mhce for for each fluoride ion concentration.



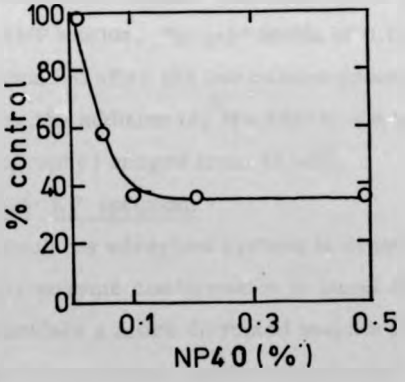


Fig. A/6 Sensitivity of adenylate cyclase activity to NP 40. Assays were performed on 4 concentrations of enzyme for each concentration of NP 40.

The assay was linear in response to enzyme concentration in the range 5-20 x 10^6 HCE and though five-fold less sensitive than the radiolabelled assays was sufficiently sensitive for the purpose.

In contrast to its effect on adenylate cyclase and succinate dehydrogenase, the detergent NP 40 stimulated NADPH-cytochrome c reductase by 50-60%. This stimulation was obtained with the lowest concentration of detergent tested (0.1%) and was not further enhanced by increasing the concentration to 1% (Fig. A/3).

(c) Adenylase cyclase activity

This activity was assayed according to Salomon, Londos and Rodbell (1974). [^{32}P]-ATP is converted to [^{32}P]-AMP by the enzyme, and this is separated from the substrates and other adenosine phosphates by sequential chromatography on columns of Dowex cation exchange resin and aluminium oxide (see Fig. A/4). ATP was clearly resolved on the Dowex column though AMP (not shown) was very close to cAMP. However, on the alumina column AMP was eluted in the 10 ml wash subsequent to cAMP elution. "Blank" levels of 0.001% of input [^{32}P] counts were normally obtained after the two column procedure, whilst recovery of product (monitored by addition of [^{3}H]-cAMP to the assay mixture immediately before chromatography) ranged from 15-40%.

Adenylate cyclase: KF optimum

Glucagon stimulates adenylate cyclase in systems in which the hormone receptor-enzyme conformation is intact in the membrane, whilst fluoride ions stimulate a more disrupted enzyme preparation (Perkins, 1973). In order to maximise the marker enzyme activity, its fluoride ion optimum was established (Fig. A/5). The optimum fluoride concentration peaked sharply at 20 mM, at which point the enzyme was 1.9-fold stimulated, and this concentration was used in all subsequent assays.

Sensitivity of adenylate cyclase to NP 40

Adenylate cyclase activity was found to be 60% sensitive to NP 40 at the concentration used in the fractionation procedure. The remaining activity was unaffected by NP 40 concentrations of up to 0.5% (Fig. A/6), but the activity was abolished at 2%.

(d) Haemagglutination activity of the virus

The sensitivity of HA activity to NP 40 was checked, and with freshly prepared red blood cells concentrations below 0.3% had no effect.

(e) Incorporation of tritiated thymidine

Cells were prelabelled for 1 or 18 h with [^{3}H]-thymidine, and fractions counted for radioactivity after TCA precipitation. There was the possibility that this might select a small population of cells which were in the process of dividing or had recently done so, and that these might be unrepresentative of the whole culture. Accordingly cell autoradiography was performed on cells which had been labelled for 1 and 18 h with [^{3}H]-thymidine. An average of total cell count and cells with grains over ten fields chosen at random showed 10% of cells were labelled in 1 h compared with 28% after 18 h. Though it seemed likely that the latter group was sufficiently large to represent all the cells and thus obviate the drawback, a further measure of DNA content was applied.

(f) Chemical assay of DNA

This measures the DNA present after repeated extraction in hot perchloric acid (Burton, 1956). The assay was investigated and found to be linear with respect to concentration of cells. It was also confirmed that the sucrose present in the detergent and nuclei fractions had no effect in the assay system.

(g) Infection with SFV

Since SFV is a cytoplasmic virus able to multiply without the need of a nucleus (S.I.T. Kennedy, personal communication) and a further test of the purity of nuclei for virological purposes relates the amounts of SFV proteins present in the nuclear fraction to that of the whole cell. This was done by pulse labelling infected cells with [^{35}S]-methionine, fractionating the cells, analysing these fractions on PAGE, scanning the resulting autoradiographs and calculating the ratio of SFV proteins in nuclear and cytoplasmic fractions.

Table A/1 Percentage distribution and recovery of markers in cell fractions

		Plasma membrane		Cytoplasm		Nuclei		
Fraction	Protein	5NT	ACA	NCR	HA	([^{3}H]-th)	DNA	SFV %
Sonicated whole cells	100	100	100	100	100	-	-	100
Cytoplasm;	ND	44	ND*	47	25	12	8	ND
Detergent extract	ND	32	ND*	52	63	5	4	ND
Nuclei	15.5	20	4	1	4	83	88	3-6 %
Total recovered	-	96	-	100	92	†(100)	†(100)	

*ND - not done † Estimates of DNA are expressed as percentages of the total recovered

5NT = 5'nucleotidase activity ACA = adenylate cyclase activity NCR = NADPH cytochrome - reductase activity

HA = haemagglutinin activity ([^{3}H]-th) = [^{3}H)-thymidine incorporated

(h) Succinate dehydrogenase activity

This enzyme activity was used as a marker for mitochondrial contamination. The method of Porteous and Clark (1965) was used in which a tetrazolium dye is reduced to formazan which is measured spectrophotometrically. The assay was found to be linear with respect to time up to 30 min and to homogenate concentration up to 12×10^6 HCE.

Sensitivity to NP 40

Succinate dehydrogenase resembled adenylate cyclase in its sensitivity to the detergent NP 40. As the concentration of NP 40 increased, activity was rapidly lost until a plateau formed at 20% residual activity for NP40 concentrations of 0.1 to 0.5%. Activity was completely abolished with 2% NP 40.

B. Results of Methods of Characterization

(a) Yield

DNA, assayed by two quite different methods, was used as the positive marker for nuclei in the fractionation. In both the chemical and the radioactivity assay there was close agreement that about 85% of the total recovered DNA was found in the nuclear fraction with 10% in the cytoplasm and traces in the detergent extract (Table A/1).

The amount of protein present in the nucleus over a large number of fractionations varied between 12-20% of the total cell protein with a mean of 15.5%.

(b) Contamination by endoplasmic reticulum

The presence of endoplasmic reticulum associated with nuclei was monitored by NADPH-cytochrome c reductase activity and by virus haemagglutinin. A mean of 1% cellular enzyme contamination and 4% virus haemagglutinin was recorded (Table A/1). Over 50% of both markers remained associated with the nuclei until after the detergent treatment, which clearly shows the importance of this step.

Table A/2

Distribution of newly synthesized SFV proteins in nuclear and cytoplasmic fractions

SFV proteins resolved on 10% phosphate PAGE		% proportion of whole cell homogenate value in nucleus
PE2	precursor of envelope protein 2	5.2
E 1 +2	envelope proteins 1 and 2	4.0
core	core protein	3.9
total	acid precipitable [^{35}S] methionine counts	6.0

Contamination by plasma membrane

This was assessed by 5' nucleotidase and adenylate cyclase activities. Though 44% of 5' nucleotidase activity was removed in the first two fractionation steps, and a further 32% in the latter two, 20% of the total activity remained stably associated with the nuclear fraction (Table A/1). This result was reproducible over a very large number of fractionations, and the ratio of 5' nucleotidase to DNA remained constant after further purification by both detergents and centrifugation on sucrose gradients. It appeared that this activity was genuinely nuclear (see below and discussion).

Adenylate cyclase activity, though considerably reduced by 0.1% NP40, was still sufficient to allow an assessment of contamination of the nuclear fraction. After allowing for the effects of NP 40 on the enzyme, there was still only 4% of the total activity present in the nuclear fraction. Activities in the dilute cytoplasmic and detergent fractions were too low to be measured, and attempts to concentrate these fractions whilst maintaining the enzyme activity proved abortive.

Mitochondrial contamination

Succinate dehydrogenase is a very commonly used marker for mitochondria. Unfortunately it is very sensitive to NP 40 thus reducing the value to be accorded these results. However, after corrections were made for this loss of activity, the degree of contamintion was estimated at less than 4%.

(c) Distribution of SFV proteins in infected cells

The proteins present in the nuclei of infected cells amounted to 4-5% of the total labelled viral proteins whilst 6% of the total TCA precipitable [^{35}S]-methionine counts were found in the nuclear fraction (Table A/2).

(d) Purification

In this method for the fractionation of chick embryo fibroblasts, a nuclear fraction is obtained which contains about 85% of the original nuclei and 15% of the whole cell protein. It is contaminated by between

1-4% of endoplasmic reticulum, less than 4% mitochondria and 4% of the total plasma membrane. Between 4 and 6% of newly synthesized proteins of a cytoplasmic virus are found in the fraction. Thus, assuming a 5% contamination overall, the purification of nuclei in this method over those in whole cells is seventeen-fold.

3. Discussion

I have thoroughly characterized a nuclear fractionation method, and shown it to yield considerably purified (17-fold) nuclei, thus enhancing the validity of studies conducted with it (Stephenson, 1974; Stephenson and Dimmock, 1974; 1975; present study).

The linearity of all the assay systems used was checked with sample material and the sensitivity of the enzymes to NP 40 was assessed and compensated for by making all solutions to the same concentration in the detergent or by allowing for the inhibition in calculating values. The enzymes seem to fall into two groups in their behaviour towards the non-ionic detergent NP 40, which is perhaps surprising since they are all membrane-bound enzymes.

5' nucleotidase and NADPH-cytochrome c reductase were unaffected or stimulated by the detergent and, in both, the values became more reproducible presumably because the particulate suspension had been rendered more homogeneous. The stimulation of NADPH-cytochrome c reductase could be accounted for on the latency prinicple in which the detergent liberates and opens up to their substrate more enzyme molecules which had been 'locked' in membrane vesicles or aggregates. Succinate dehydrogenase and adenylate cyclase on the other hand are both considerably inhibited by the presence of the detergent, presumably due to the sensitivity of their conformation to their membrane interaction which is affected by the treatment. Both these enzymes display a two-step process in their inhibition by NP 40. Very low concentrations are sufficient to effect the initial reduction in enzyme activity, the remainder of which (35% of adenylate cyclase, 20% of SDH) was insensitive to the detergent at concentrations from 0.1-0.5%. An explanation for this phenomenon is that the residual activity represents the enzyme in

solution and that the smallest concentration of detergent (0.05%) is sufficient to cause solubilization.

The markers for endoplasmic reticulum were in good agreement showing that the detergent step appeared to be essential in removing over half of this component, and that the final residual ER in the nuclear fraction was 4% or less. Yields of DNA monitored by [^{3}H]-thymidine labelling and chemical analysis were also in close agreement. The remaining DNA in cytoplasm and detergent extracts (10-15%) probably represents mitochondrial DNA and ruptured nuclei. Plasma membrane enzymes gave very different values for contamination of the nuclear fraction. 5' nucleotidase gave a regular and reproducible 20% contamination. Further purification of nuclei by repeated detergent treatment, pelleting and banding on sucrose gradients lowered the yield but failed to improve the ratio between this enzyme and the DNA content. Conversely, adenylate cyclase gave a value which corresponded to the general levels of contamination recorded from other cell compartments. There have been reports of a nuclear 5' nucleotidase activity in a variety of cells (de Pierre and Karnovsky, 1973; Bosmann, Hagopian and Eylar, 1968; Avruch and Wallach, 1971) in addition to the plasma membrane enzyme. Thus I conclude that the stable level of 5' nucleotidase is, in fact, a genuine nuclear enzymic activity. Confirmation of this would involve the measurement of plasma membrane contamination by another criterion such as tagging the intact plasma membrane with [I^{125}] by the lactoperoxidase method prior to fractionation. Perhaps a valid criticism of this multiple marker approach to subcellular fractionation is the difficulty of knowing at what point to stop the characterization of fractions by yet more methods.

4. Conclusion

The conventional fractionation method characterised in these studies produced highly purified nuclei in a simple operation of 60-75 mins. However, in the rapid growth cycle of influenza virus with its ability to complete certain steps even at 4°, a faster and yet simpler method was

required. This was provided by the nuclear monolayer technique which allowed rapid, concentrated nuclear and cytoplasmic fractions of CEF cell monolayers to be prepared, and was used in the majority of the cell fractionations reported in this study.

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